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EVALUATION OF SEVERAL BIOLOGICAL MONITORING TECHNIQUES FOR HAZARD ASSESSMENT OF POTENTIALLY CONTAMINATED WASTEWATER AND GROUNDWATER

VOLUME 3 - OLD O-FIELD GROUNDWATER

FINAL REPORT

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The toxicity of contaminated Old O-Field (Edgewood Area of Aberdeen Proving Ground) groundwater and the reduction and/or elimination of toxicity by various treatment processes were evaluated. The study was divided into a bench scale and pilot scale study. The bench scale studies consisted of 48-h definitive acute toxicity tests run with daphnid neonates (Daphnia magna) and juvenile fathead minnows (Pimephales promelas) exposed to untreated Old O-Field groundwater and groundwater treated by 1) metals precipitation, 2) UV oxidation (H_2O_2), 3) carbon adsorption, and 4) carbon adsorption/biological sludge. The pilot scale studies consisted of 1) several 96-h definitive acute toxicity tests run with two freshwater and two saltwater invertebrates and fish and 2) Ames mutagenicity assays. Acute toxicity tests were run on untreated Old O-Field groundwater and groundwater treated by 1) metals precipitation, 2) UV oxidation (H_2O_2), 3) air-stripping, and 4) carbon adsorption during the pilot scale study. The freshwater invertebrate and fish used in the study were daphnid neonates and juvenile fathead minnows, respectively. The saltwater

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invertebrate and fish were juvenile mysids (Mysidopsis bahia) and juvenile sheepshead minnows (Cyprinodon variegatus). Ames tests were run on 1) untreated groundwater, 2) UV oxidation-treated groundwater, and 3) carbon-treated groundwater.

Untreated Old-O-Field groundwater was acutely toxic to daphnids and fathead minnows in two separate bench scale tests. Likewise, untreated groundwater was acutely toxic during the pilot scale test to daphnids, fathead minnows, mysids, and sheepshead minnows. The pH of the untreated groundwater, which was 5.7 to 5.8 in the bench scale tests and approximately 3.2 in the pilot scale test, was a factor contributing to toxicity; pH alone was not responsible for all of the toxicity. Untreated groundwater obtained during the pilot scale test and subsequently concentrated 10-fold was mutagenic in three out of four Ames trials. Untreated groundwater, which was not concentrated, was not mutagenic during the pilot scale study.

The following bench scale treatment technologies eliminated and/or reduced the acute toxicity of the untreated groundwater: carbon adsorption and carbon adsorption/biological sludge. UV oxidation and metals precipitation eliminated toxicity in one out of two bench scale tests. In the pilot scale studies, all of the following treatment technologies eliminated the acute toxicity of the groundwater: chemical precipitation, UV oxidation, air stripping, and carbon treatment. No mutagenic activity was found during the pilot scale study in unconcentrated or concentrated (10X) UV oxidation- or carbon-treated groundwater.

EXECUTIVE SUMMARY

The toxicity of contaminated Old O-Field (Edgewood Area of Aberdeen Proving Ground) groundwater and the reduction and/or elimination of toxicity by various treatment processes were evaluated. The study was divided into two phases. The first phase was designed to determine the effectiveness of several bench scale treatment technologies to reduce toxicity of the contaminated groundwater. The second phase was designed to determine the effectiveness of an on-site pilot scale treatment system composed of several treatment components.

The bench scale studies consisted of 48-h definitive acute toxicity tests run with daphnid neonates (Daphnia magna) and juvenile fathead minnows (Pimephales promelas) exposed to untreated Old O-Field groundwater and groundwater treated by 1) metals precipitation, 2) UV oxidation (H_2O_2), 3) carbon adsorption, and 4) carbon adsorption/biological sludge. The pilot scale studies consisted of 1) several 96-h definitive acute toxicity tests run with two freshwater and two saltwater invertebrates and fish and 2) Ames mutagenicity assays. Acute toxicity tests were run on untreated Old O-Field groundwater and groundwater treated by 1) metals precipitation, 2) UV oxidation (H_2O_2), 3) air stripping, and 4) carbon adsorption during the pilot scale study. The freshwater invertebrate and fish used in the study were daphnid neonates and juvenile fathead minnows, respectively. The saltwater invertebrate and fish were juvenile mysids (Mysidopsis bahia) and juvenile sheepshead minnows (Cyprinodon variegatus). Ames tests were run on 1) untreated groundwater, 2) UV oxidation-treated groundwater, and 3) carbon-treat groundwater.

Untreated Old O-Field groundwater was acutely toxic to daphnids and fathead minnows in two separate bench scale tests. Likewise, untreated groundwater was acutely toxic during the pilot scale test to daphnids, fathead minnows, mysids, and sheepshead minnows. The pH of the untreated groundwater, which was 5.7 to 5.8 in the bench scale tests and ≈3.2 in the pilot scale test, was a factor contributing to toxicity; pH alone was not responsible for all of the toxicity. Untreated groundwater obtained during the pilot scale test and subsequently concentrated 10-fold was mutagenic in three out of four Ames trials. Untreated groundwater, which was not concentrated, was not mutagenic during the pilot scale study.

The following bench scale treatment technologies eliminated and/or reduced the acute toxicity of the untreated groundwater: carbon adsorption and carbon adsorption/biological sludge. UV oxidation and metals precipitation eliminated toxicity in one out of two bench scale tests. In the pilot scale studies, all of the following treatment technologies eliminated the acute toxicity of

the groundwater: chemical precipitation, UV oxidation, air stripping, and carbon treatment. No mutagenic activity was found during the pilot scale study in unconcentrated or concentrated (10X) UV oxidation- or carbon-treated groundwater.

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SECTION 1

INTRODUCTION

The Directorate of Safety, Health, and Environment at the Aberdeen Proving Ground asked the Health Effects Research Division/Research Methods Branch of the U.S. Army Biomedical Research and Development Laboratory to provide biomonitoring assessments of contaminated Old O-Field (Edgewood Area of Aberdeen Proving Ground) groundwater treated by various methods to reduce and/or eliminate toxicity. The study was divided into two phases. The first phase was designed to determine the effectiveness of several bench scale treatment technologies to reduce toxicity of the contaminated groundwater. The second phase was designed to determine the effectiveness of an on-site pilot scale treatment system composed of several treatment components.

The bench scale studies consisted of two series of 48-h definitive acute toxicity tests run with daphnid neonates (Daphnia magna) and juvenile fathead minnows (Pimephales promelas) exposed to untreated Old O-Field groundwater and groundwater treated by the processes described below. The first series of tests were performed during the period January 9-25, 1991, on samples supplied by personnel from ICF Technology Incorporated (ICF) which were treated at the Old O-Field site. A second series of bioassays were conducted during the period March 20-25, 1991, on samples treated by Peroxidation Systems, Inc. (PSI), Tucson, Arizona. The second series was initiated because the UV oxidation (H_2O_2) treatment in the first series of tests increased toxicity relative to the untreated groundwater. All untreated groundwater samples were obtained from composite samples of Old O-Field well numbers OF6A and OF6B mixed 1:2 (LaCaria et al., 1991).

Groundwater was treated by the following processes during the first series of bench scale studies: 1) metals precipitation, 2) UV oxidation (H_2O_2), 3) carbon adsorption, and 4) carbon adsorption/biological sludge. Groundwater was shipped to PSI and treated as follows during the second series of the bench scale studies: 1) metals precipitation and 2) UV oxidation (H_2O_2). A sample of Johns Hopkins University Applied Physics Laboratory (JHU/APL) diluent water was sent to PSI, treated by UV oxidation (H_2O_2), and returned to JHU/APL for acute toxicity analysis. The JHU/APL sample served as an UV oxidation control. An untreated groundwater sample was also shipped from PSI back to JHU/APL for acute toxicity analysis.

The pilot scale studies consisted of 1) several 96-h definitive acute toxicity tests run with two freshwater and two

saltwater invertebrates and fish during the period April 26 to May 6, 1991, and 2) Ames mutagenicity assays. Acute toxicity tests were run on untreated Old O-Field groundwater and groundwater treated by 1) metals precipitation, 2) UV oxidation (both a "best" and "worse" treatment case), 3) air stripping, and 4) carbon adsorption. The freshwater invertebrate and fish used in the study were daphnid neonates and juvenile fathead minnows, respectively. The saltwater invertebrate and fish were juvenile mysids (Mysidopsis bahia) and juvenile sheepshead minnows (Cyprinodon variegatus). Ames tests were run on 1) untreated groundwater, 2) UV oxidation ("best" treatment case), and 3) carbon treatment.

The untreated groundwater samples were supplied by ICF personnel from a composite sample of Old O-Field well numbers OF6B, OF13C, and OF17B mixed ≈30, 40, and 30%, respectively (LaCaria et al., 1991). Effluent from the various groundwater treatment processes were also supplied by personnel from ICF. Complete chemical analyses were also performed on the untreated and all treated groundwater samples. The acute toxicity tests, Ames tests, and chemical analyses were performed on subsamples taken from the same untreated and treated groundwaters samples.

SECTION 2

MATERIALS AND METHODS

2.1 Bench Scale Studies

All 48-h bioassays were conducted by definitive static renewal acute toxicity procedures recommended by the U.S. EPA (EPA) for measuring the acute toxicity of effluents to freshwater organisms (Peltier and Weber, 1985). Neonate daphnids <24 h old at the start of the test were used in all bioassays. Juvenile fathead minnows used in the first series of tests were 35-40 d old; the fish used in the second series of tests were 16-18 d old. The specific test conditions for the daphnids and fathead minnows are given in Tables 1 and 2, respectively.

Approximately 10 L of untreated and treated groundwater were obtained in glass containers by personnel from both ICF (Test Series 1) and PSI (Test Series 2) and shipped iced in insulated containers via Federal Express to JHU/APL. The bioassays were initiated the day the samples were received. A preliminary range finding test was run with the first untreated groundwater sample only before the definitive test was initiated. The dates the samples were received at JHU/APL are given in Tables 5 and 6.

All samples were warmed to the test temperature of 20°C before being used in the tests. The samples were not aerated prior to the start of a test or renewal of test material at 24 h unless the dissolved oxygen concentrations were below 40% saturation. The test organisms were obtained from in-house cultures at JHU/APL. Unchlorinated JHU/APL well water was used for all cultures and test dilutions.

Dissolved oxygen and pH were measured in all test concentrations at time 0 h and 24 h on day 1 and again at time 0 h and 24 h when the material was renewed at 24 h. Hardness, alkalinity, conductivity, and temperature were measured at the same frequency in only the control and 100% treatments. Routine water quality was determined by procedures given in Standard Methods (APHA et al., 1989). The chemical composition of the various treatments was determined by independent vendors under the direction of ICF (LaCaria et al., 1991). The chemical composition data are not included in this report since they are presented in LaCaria et al. (1991).

All LC50s were determined by the moving average angle method as recommended by EPA (Peltier and Weber, 1985). The LC50 values and their 95% confidence limits are reported as percent groundwater by volume. The moving average angle method for determining LC50s was performed by a computer program developed by Stephan (1978).

2.2 Pilot Scale Studies

2.2.1 Acute Toxicity Tests

All 96-h bioassays were conducted by definitive static renewal acute toxicity procedures recommended by EPA for measuring the acute toxicity of effluents to freshwater and saltwater organisms (Peltier and Weber, 1985). The following deviations from the U.S. EPA method occurred during the tests: 1) all the daphnid bioassays were run for 96 h rather than 48 h; thus, the daphnids were fed daily (0.2 mL of 1 x 10^9 Selenastrum capricornutum cells/50 mL solution containing 10 daphnids) at each renewal during the test and 2) all organisms were run at 25°C rather than 20°C because it is the recommended test temperature in the forthcoming 4th edition of the EPA testing manual (Peltier, personal communication).

Neonate daphnids <24 h old at the start of the test were used in all bioassays. The juvenile mysids were 3-4 d old at the start of the tests. Both juvenile fathead minnows and sheepshead minnows were 8-10 d old at the beginning of the bioassays. The specific test conditions for the daphnids, fathead minnows, mysids, and sheepshead minnows are given in Tables 1-4, respectively.

Approximately 11 L of untreated and treated groundwater were obtained in glass containers by personnel from ICF, packed in ice, and transported back to the JHU/APL toxicology laboratory in insulated containers. The following samples were run within 24 h of receipt of the material: 1) untreated Old O-Field groundwater and groundwater treated by 2) metals precipitation, 3) UV oxidation, 4) air stripping, and 5) carbon adsorption. Two UV oxidation samples, which were treated as the "best" UV oxidation treatment (Test No. 1) and the "worse" UV oxidation treatment (Test No. 2), were analyzed for toxicity. Detailed information on the equipment, operating procedures, system performance, etc. of each type of treatment process is given in LaCaria et al. (1991); thus, it will not be repeated in this report. The ICF sample identification numbers for all samples tested were as follows:

Untreated groundwater	APG-OOF-PS-2A
Metals precipitation	APG-OOF-PS-2B
UV oxidation (Test No. 1)	APG-OOF-PS-1C4
UV oxidation (Test No. 2)	APG-OOF-PS-2C4
Air stripping	APG-OOF-PS-1D
Carbon	APG-OOF-PS-1I

The test effluents for all saltwater bioassays were salted with Instant Ocean to 20 ppt the day the samples arrived at JHU/APL and allowed to equilibrate for 18-24 h before the

saltwater organisms were placed in the test effluents. All samples were warmed to the test temperature of 25°C before being used in the tests. The samples were not aerated prior to the start of a test or renewal of test material at 24 h unless the dissolved oxygen concentrations were below 40% saturation.

The test organisms for all tests were obtained from in-house cultures at JHU/APL. Unchlorinated JHU/APL well water was used for all freshwater cultures and test dilutions. Filtered Chesapeake Bay water, which ranged from 8-12 ppt, was salted with Instant Ocean to 20 ppt and used for all saltwater culturing and test dilutions.

Dissolved oxygen and pH were measured in all test concentrations at the beginning and end of each 24 h renewal period up to 96 h. Temperature and salinity were measured in only the control and 100% treatments at each renewal. The chemical composition of the various treatments was determined by independent vendors under the direction of ICF (LaCaria et al., 1991). The chemical composition data are not included in this report since they presented in LaCaria et al. (1991).

All LC50s were determined by the moving average angle method as recommended by EPA for measuring the acute toxicity of effluents to freshwater and marine organisms (Peltier and Weber, 1985). All LC50 values and their 95% confidence limits are reported as percent groundwater by volume. The moving average angle method for determining LC50s was performed by a computer program developed by Stephan (1978).

2.2.2 Mutagenicity Tests

Salmonella/mammalian-microsome reverse mutation assays (Ames test) were conducted on 1) untreated groundwater, and groundwater treated by 2) UV oxidation ("best" treatment case), and 3) carbon. Assays were conducted on both unconcentrated and concentrated (10X via XAD-2 resin extracts) samples of untreated groundwater, UV oxidation-treated groundwater, and carbon-treated groundwater. Groundwater treated by chemical precipitation and air stripping were not evaluated for mutagenic activity. The Ames mutagenicity assays were conducted by Hazelton Washington, Inc., Kensington, Maryland.

Thirty-one liters (1 L for the unconcentrated sample and 30 L for the 10X sample) of each material were obtained in glass containers by personnel from ICF, packed in ice, and transported to Hazelton Washington, Inc. in insulated containers. The samples were taken to Hazelton Washington, Inc. the same day the samples were collected. The unconcentrated samples were analyzed by Hazelton Washington, Inc. Protocol No. HWA Protocol 401W, Edition 16; the 10X samples were analyzed by Protocol No. HWA Protocol 401X, Edition 17.

The experimental procedures for the unconcentrated and 10X tests are given in the reports of the studies presented in the Appendixes of this report. Briefly, the mutagenicity assay evaluated the test material for its ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor-induced rat liver. The tester strains used in the assays were TA98 and TA100. The assays were conducted using two plates per dose level in the presence of microsomal enzymes. Six dose levels of the test material were tested in both the presence and absence of S9 along with appropriate vehicle controls (three plates per dose), negative controls, and positive controls. Resins controls were also run for the 10X samples. The doses tested in the 10X assays varied based on the amount of extractable organics recovered from the test material.

SECTION 3

RESULTS AND DISCUSSION

3.1 Bench Scale Studies

The 48-h LC50s for both the first and second series of tests are summarized in Tables 5 and 6 for the daphnids and fathead minnows, respectively. The mortality data for all treatments are summarized in Table 11. The water quality for all test conditions at each test concentration at time 0 h, 24 h old, renewal of test solutions at 24 h (24 h new) and at the end of the test (48 h old) are given in Table 12. The temperature for all tests was 20 ± 0.3°C.

3.1.1 Test Series 1

Untreated groundwater and groundwater treated by UV oxidation during the first set of tests were toxic to both the daphnids and fathead minnows. The 48-h LC50s for daphnids exposed to untreated groundwater and groundwater treated by UV oxidation were 33.4 and <10% groundwater by volume, respectively (Table 5). The 48-h LC50s for fathead minnows exposed to untreated groundwater and groundwater treated by UV oxidation were 50.8 and 12.9% groundwater by volume, respectively (Table 6). The UV oxidation-treated groundwater was more toxic than untreated groundwater to both daphnids and fathead minnows.

The metal precipitated-groundwater eliminated the acute toxicity to both daphnids and fathead minnows. Mortality ranged from 10-30% for daphnids in the carbon adsorption-treated groundwater; no mortality occurred to fathead minnows (Table 11; p. 37).

The initial pH of the untreated groundwater, which was used in the acute toxicity tests to derive the LC50s, was ≈5.8 (Table 11; p. 39). Because of the low pH, two additional treatments were run with daphnids and fathead minnows using 100% untreated groundwater adjusted to pH 7 by NaOH and Na_2CO_3. The untreated groundwater adjusted with NaOH and Na_2CO_3 still killed 85 and 75% of the daphnids, respectively (Table 11; p. 30). The untreated groundwater adjusted with NaOH and Na_2CO_3 killed 35 and 50%, respectively, of the fathead minnows.

The pH of the UV oxidized-treated groundwater was also very low. UV oxidized groundwater with the pH adjusted from 4.1 to 7 with Na_2CO_3 still caused 100% mortality to both the daphnids and fathead minnows (Table 11; p. 34).

The initial pH of the metals-precipitated groundwater was

≈10.1; therefore, a concurrent test with the pH lowered via HCl to 7 was conducted (Table 12; p. 41). As was the case at pH 10.1, the metals-treated groundwater at 7 was not toxic to daphnids or fathead minnows.

3.1.2 Test Series 2

The untreated groundwater during the second set of tests was also toxic to both the daphnids and fathead minnows. The 48-h LC50s for daphnids and fathead minnows exposed to untreated groundwater were 57.5 and 56.8% groundwater by volume, respectively (Tables 5 and 6). The untreated groundwater was less toxic to both the daphnids and fathead minnows during the second series of tests relative to the first series of bioassays. The 48-h LC50 for daphnids during the first test was 33.4 in contrast to 57.5% groundwater by volume in the second test. The 48-h LC50 for the fathead minnows during the first test was 50.8 in contrast to 56.8% groundwater by volume in the second test.

The initial pH of the untreated groundwater during the second series of tests, which was used in the acute toxicity tests to derive the LC50s, was ≈5.7 (Table 12; p. 40). An additional treatment was run with daphnids and fathead minnows using 100% untreated groundwater adjusted to pH 7 by Na_2CO_3. The untreated groundwater adjusted with Na_2CO_3 still killed 50 and 30%, respectively, of the daphnids and fathead minnows (Table 11; p. 31). Similar mortality values were obtained in pH adjusted untreated groundwater in the first series of bioassays.

In contrast to the first series of bioassays, the metals precipitated-treated groundwater was toxic to both species during the second series of tests. The 48-h LC50s were 64.7% groundwater by volume for both the daphnid and fathead minnow. No mortality occurred to either species in the metals-precipitated groundwater in the first series of bioassays. The initial pH of the 100% metals-precipitated groundwater during the first test was 10.1 (Table 12; p. 41). In the case of the fathead minnow during the second test, the toxicity may be a function of high pH. The initial pH of the 100% metals-precipitated groundwater was 10.9 (Table 12; p. 42). When the pH was adjusted by HCl to ≈7.1, daphnid mortality was reduced from 100% to 50% (Table 11; p. 33). No mortality occurred to fathead minnows in 100% metals-precipitated groundwater.

The UV oxidation-treated groundwater and UV oxidation-treated JHU/APL diluent water samples were not toxic to the daphnid or fathead minnow during the second series of bioassays. In contrast to the first series of tests, the UV oxidation-treated groundwater in the second series of tests was buffered to a pH of ≈7.5 by the vendor before the UV oxidation-treated groundwater was sent for toxicity analysis. As discussed above,

the pH adjusted UV oxidation-treated groundwater in the first series of bioassays was still toxic to both species.

3.2 Pilot Scale Studies

3.2.1 Acute Toxicity Tests

The 96-h LC50s are summarized in Tables 7-10 for daphnids, fathead minnows, mysids, and sheepshead minnows, respectively. The daphnid and fathead minnow mortality data for all treatments are summarized in Table 13. The mortality data for the mysids and sheepshead minnows are summarized in Table 14. The pH, dissolved oxygen, and salinity data for all test conditions at each test concentration at time 0 h, 24 h later (old), and renewal of test solutions every 24 h (new) are given in Table 15. The temperature for all tests was 25 ± 0.2°C.

The untreated groundwater was toxic to all four test species. The 96-h LC50s for daphnids, fathead minnows, mysids, and sheepshead minnows were 33.4, 42.4, 38.8, and 41.3% groundwater by volume, respectively (Tables 7-10). No significant mortality (5% or less in 100% test material by volume) occurred to any test species in the groundwater treated by metals precipitation, UV oxidation (Test Nos. 1 and 2), air stripping, or carbon adsorption.

The pH of the untreated groundwater, which was used in the acute freshwater toxicity tests to derive the LC50s, was ≈3.2 (Table 15; p. 60). The pH of the untreated groundwater salted to 20 ppt for use in the saltwater bioassays was ≈6.2 (Table 15; p. 61). Because of the low pH in the untreated groundwater, an attempt was made to adjust an undiluted sample to ≈7 to run as a pH control. However, upon the addition of base (1N Na_2CO_3), large amounts of material precipitated out of solution. A decision was made not to run a pH control because 1) we were not certain what materials may have precipitated out of solution and 2) the EPA test method does not allow any adjustments, except for dissolved oxygen (<40% saturation) and salinity (saltwater tests only), for actual determination of the LC50.

All daphnids and fathead minnows died in the 100% untreated groundwater which had a pH of ≈3.2 (Table 15; p. 48). A pH of ≈3.2 would kill all organisms even if no toxicants were present. All daphnids and fathead minnows also died in the 56% dilution which had a pH of 6.0. Although a pH of 6.0 may be stressful to daphnids and fathead minnows, it will not kill the organisms in 96 h (Peltier and Weber, 1985). The EPA test manual allows a pH as low as 6.0 to be used for acute toxicity tests. Thus, one may conclude that there is toxicity in the 56% dilution which is not directly related to pH.

The same arguments concerning pH may be made for the mysids and sheepshead minnows. Undiluted groundwater adjusted to 20 ppt caused 100% mortality to both saltwater species (Table 14; p. 54) at a pH of 6.2 which is within the range allowed by EPA for acute toxicity testing. A 56% dilution killed 100% of the mysids and 85% of the sheepshead minnows at a pH of 6.5. Thus, one can conclude that the toxicity present is not related to pH alone.

2.2.2 Mutagenicity Tests

The results of the unconcentrated and 10X Ames tests for the untreated groundwater are given in Appendixes 1 and 2, respectively. The unconcentrated and 10X UV oxidation treated-groundwater data are given in Appendixes 3 and 4; the carbon-treated groundwater results are presented in Appendixes 5 and 6. The results of all samples assayed during the study can be briefly summarized as follows. With the exception of the concentrated (10X) untreated groundwater sample, none of the samples caused a positive increase in the numbers of histidine revertants per plate with tester strains TA98 and TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

The concentrated (10X) untreated groundwater did cause a positive increase in the number of histidine revertants per plate with tester strain TA98 in the presence of S9 in two independent trials (3.6- and 4.5-fold). A positive increase was also found with tester strain TA100 in the presence of S9 (2.0-fold) in an initial experiment. However, in the confirmatory assay, the positive increase could not be reproduced. No positive increases were observed with either tester strain in the absence of S9.

SECTION 4

CONCLUSIONS

Untreated Old O-Field groundwater was acutely toxic (48-h LC50s) to daphnids and fathead minnows in two separate bench scale tests. Likewise, untreated groundwater was acutely toxic (96-h LC50s) during the pilot scale test to daphnids, fathead minnows, mysids, and sheepshead minnows. The initial pH of the untreated groundwater, which was 5.7 to 5.8 in the bench scale tests and ≈3.2 in the pilot scale test, was a factor contributing to toxicity; pH alone did not account for all of the toxicity. Untreated groundwater obtained during the pilot scale test and subsequently concentrated 10-fold was mutagenic in three out of four Ames trials. Untreated groundwater, which was not concentrated, was not mutagenic during the pilot scale study.

The following bench scale treatment technologies eliminated and/or reduce the acute toxicity of the untreated groundwater: carbon adsorption and carbon adsorption/biological sludge. UV oxidation and metals precipitation eliminated toxicity in one out of two bench scale tests. In the pilot scale studies, all of the following treatment technologies eliminated the acute toxicity of the groundwater: chemical precipitation, UV oxidation, air stripping, and carbon treatment. No mutagenic activity was found during the pilot scale study in unconcentrated or concentrated (10X) UV oxidation- or carbon-treated groundwater.

SECTION 5

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TABLE 1. EPA RECOMMENDED TEST CONDITIONS FOR DAPHNIDS (*DAPHNIA MAGNA*).

1.	Temperature:	20 ± 2°C[a]
2.	Light quality:	Ambient laboratory illumination
3.	Light intensity:	50-100 footcandles (ambient laboratory levels)
4.	Photoperiod:	16 h light: 8 h dark
5.	Size of test vessel:	100 mL beaker
6.	Volume of test solution:	50 mL
7.	Age of test animals:	1-24 h (neonates)
8.	Number of animals per test vessel:	10
9.	Number of replicate test vessels per concentration:	2
10.	Total number of organisms per concentration:	20
11.	Feeding regime:	Feeding not required during first 48 h. For longer tests, feed every other day
12.	Aeration:	None, unless DO concentration falls below 40% of saturation, at which time start gentle aeration
13.	Dilution water:	Receiving water or laboratory groundwater
14.	Test duration:	48 h - static 96 h - static renewal
15.	Effect measured:	Mortality - no movement of body or appendages on gentle prodding

[a] Pilot scale tests conducted at 25 ± 2°C to reflect change recommended in the upcoming 4th ed. EPA acute testing manual.

TABLE 2. EPA RECOMMENDED TEST CONDITIONS FOR FATHEAD MINNOWS (*PIMEPHALES PROMELAS*).

1.	Temperature:	20 ± 2°C[a]
2.	Light quality:	Ambient laboratory illumination
3.	Light intensity:	50-100 footcandles (ambient laboratory levels)
4.	Photoperiod:	16 h light: 8 h dark
5.	Size of test vessel:	1 L beaker
6.	Volume of test solution:	0.75 L
7.	Age of test animals:	1-90 days
8.	Number animals per test vessel:	10 (not to exceed loading limits)
9.	Number of replicate test vessels per concentration:	2
10.	Total number of organisms per concentration:	20
11.	Feeding regime:	Feeding not required
12.	Aeration:	None, unless DO concentration falls below 40% of saturation, at which time start gentle aeration
13.	Dilution water:	Receiving water or laboratory groundwater
14.	Test duration:	48 h - static 96 h - static renewal or continuous slow
15.	Effect measured:	Mortality - no movement

[a] Pilot scale tests conducted at 25 ± 2°C to reflect change recommended in the upcoming 4th ed. EPA acute testing manual.

TABLE 3. EPA RECOMMENDED TEST CONDITIONS FOR MYSIDS (MYSIDOPSIS BAHIA).

1.	Salinity:	20-30 ppt
2.	Temperature:	20 ± 2°C[a]
3.	Light intensity:	50-100 footcandles (ambient laboratory levels)
4.	Photoperiod:	16 h light: 8 h dark
5.	Size of test vessel:	250 mL glass beaker
6.	Volume of test solution:	200 mL per rep
7.	Age of test animals:	1-5 days
8.	Number of animals per test vessel:	10
9.	Number of replicate test vessels per concentration:	2
10.	Total number of organisms per concentration:	20
11.	Feeding regime:	Feed 150 24-h old brine shrimp nauplii per mysid twice daily
12.	Aeration:	None, unless DO concentration falls below 40% of saturation, at which time start gentle aeration
13.	Dilution water:	Natural seawater or artificial sea salts
14.	Test duration:	48 h - static 96 h - static renewal
15.	Effect measured:	Mortality - no movement of body or appendages on gentle prodding

[a] Pilot scale tests conducted at 25 ± 2°C to reflect change recommended in the upcoming 4th ed. EPA acute testing manual.

TABLE 4. EPA RECOMMENDED TEST CONDITIONS FOR SHEEPSHEAD MINNOWS (CYPRINODON VARIEGATUS).

1.	Salinity:	20-30 ppt
2.	Temperature:	20 ± 2°C[a]
3.	Light intensity:	50-100 footcandles (ambient laboratory levels)
4.	Photoperiod:	14 h light: 10 h dark
5.	Size of test vessel:	1 L
6.	Volume of test solution:	0.75 L
7.	Age of test animals:	1-90 days
8.	Number of animals per test vessel:	10 (not to exceed loading limits)
9.	Number of replicate test vessels per concentration:	2
10.	Total number of organisms per concentration:	20
11.	Feeding regime:	Feeding not required
12.	Aeration:	None, unless DO concentration falls below 40% of saturation, at which time start gentle aeration
13.	Dilution water:	Natural seawater or artificial sea salts
14.	Test duration:	48 h - static 96 h - static renewal or continuous flow
15.	Effect measured:	Mortality - no movement

[a] Pilot scale tests conducted at 25 ± 2°C to reflect change recommended in the upcoming 4th ed. EPA acute testing manual.

TABLE 5. SUMMARY OF THE OLD O-FIELD GROUNDWATER BENCH SCALE 48-H LC50 VALUES AND ASSOCIATED 95% CONFIDENCE LIMITS OF NEONATE DAPHNIDS.

Type of Test	Date[a]	48-h LC50 (95% Confidence Limits) (Percent Groundwater by Volume)
Untreated (Test No. 1)	1/09/91 1/12/91	33.4 (27.6 - 39.1)
Untreated (Test No. 2)	3/21/91 3/23/91	57.5 (51.8 - 64.2)
Metals Precipitation (Test No. 1)	1/18/91 1/20/91	No mortality in 100% effluent
Metals Precipitation (Test No. 2)	3/21/91 3/23/91	64.7 (59.6 - 71.2)
UV Oxidation (Test No. 1)	1/18/91 1/20/91	<10
UV Oxidation (Test No. 2)	3/23/91 3/25/91	No mortality in 100% effluent
UV Oxidation-JHU/APL Diluent Water	3/23/91 3/25/91	No mortality in 100% effluent
Carbon Adsorption	1/16/91 1/18/91	<50% mortality in 100% effluent
Carbon Adsorption/ Biological Sludge	1/23/91 1/25/91	No significant mortality in 100% effluent

[a] The upper date for each sample is the date the sample was received at JHU/APL; the lower date is the date the bioassay was completed at JHU/APL.

TABLE 6. SUMMARY OF THE OLD O-FIELD GROUNDWATER BENCH SCALE 48-H LC50 VALUES AND ASSOCIATED 95% CONFIDENCE LIMITS OF JUVENILE FATHEAD MINNOWS.

Type of Test	Date[a]	48-h LC50 (95% Confidence Limits) (Percent Groundwater by Volume)
Untreated (Test No. 1)	1/09/91 1/12/91	50.8 (44.0 - 59.9)
Untreated (Test No. 2)	3/21/91 3/23/91	56.8 (50.9 - 63.5)
Metals Precipitation (Test No. 1)	1/18/91 1/20/91	No mortality in 100% effluent
Metals Precipitation (Test No. 2)	3/21/91 3/23/91	64.7 (59.6 - 71.1)
UV Oxidation (Test No. 1)	1/18/91 1/20/91	12.9 (0.44 - 14.63)
UV Oxidation (Test No. 2)	3/23/91 3/25/91	No mortality in 100% effluent
UV Oxidation-JHU/APL Diluent Water	3/23/91 3/25/91	No mortality in 100% effluent
Carbon Adsorption	1/16/91 1/18/91	No mortality in 100% effluent
Carbon Adsorption/ Biological Sludge	1/23/91 1/25/91	No mortality in 100% effluent

[a] The upper date for each sample is the date the sample was received at JHU/APL; the lower date is the date the bioassay was completed at JHU/APL.

TABLE 7. SUMMARY OF THE OLD O-FIELD GROUNDWATER PILOT SCALE 96-H LC50 VALUES AND ASSOCIATED 95% CONFIDENCE LIMITS OF NEONATE DAPHNIDS.

Type of Test	Date[a]	96-h LC50 (95% Confidence Limits) (Percent Groundwater by Volume)
Untreated	4/26/91 5/01/91	33.4 (28.1 - 38.6)
Chemical Precipitation	4/26/91 5/01/91	No mortality in 100% effluent
UV Oxidation (Test No. 1)	4/26/91 5/01/91	No mortality in 100% effluent
UV Oxidation (Test No. 2)	4/27/91 5/03/91	No mortality in 100% effluent
Air Stripping	5/01/91 5/06/91	No mortality in 100% effluent
Carbon Treatment	5/01/91 5/06/91	No mortality in 100% effluent

[a] The upper date for each sample is the date the sample was received at JHU/APL; the lower date is the date the bioassay was completed at JHU/APL.

TABLE 8. SUMMARY OF THE OLD O-FIELD GROUNDWATER PILOT SCALE 96-H LC50 VALUES AND ASSOCIATED 95% CONFIDENCE LIMITS OF JUVENILE FATHEAD MINNOWS.

Type of Test	Date[a]	96-h LC50 (95% Confidence Limits) (Percent Groundwater by Volume)
Untreated	4/26/91 5/01/91	42.3 (36.5 - 49.1)
Chemical Precipitation	4/26/91 5/01/91	No mortality in 100% effluent
UV Oxidation (Test No. 1)	4/26/91 5/01/91	No mortality in 100% effluent
UV Oxidation (Test No. 2)	4/27/91 5/03/91	5% mortality in 100% effluent
Air Stripping	5/01/91 5/06/91	No mortality in 100% effluent
Carbon Treatment	5/01/05 5/06/91	No mortality in 100% effluent

[a] The upper date for each sample is the date the sample was received at JHU/APL; the lower date is the date the bioassay was completed at JHU/APL.

TABLE 9. SUMMARY OF THE OLD O-FIELD GROUNDWATER PILOT SCALE 96-H LC50 VALUES AND ASSOCIATED 95% CONFIDENCE LIMITS OF JUVENILE MYSIDS.

Type of Test	Date[a]	96-h LC50 (95% Confidence Limits) (Percent Groundwater by volume)
Untreated	4/26/91 5/01/91	38.8 (33.3 - 44.8)
Chemical Precipitation	4/26/91 5/01/91	No mortality in 100% effluent
UV Oxidation (Treatment No. 1)	4/26/91 5/01/91	No mortality in 100% effluent
UV Oxidation (Treatment No.2)	4/27/91 5/03/91	No mortality in 100% effluent
Air Stripping	5/01/91 5/06/91	No mortality in 100% effluent
Carbon Treatment	5/01/91 5/06/91	No mortality in 100% effluent

[a] The upper date for each sample is the date the sample was received at JHU/APL; the lower date is the date the bioassay was completed at JHU/APL.

TABLE 10. SUMMARY OF THE OLD O-FIELD GROUNDWATER PILOT SCALE 96-H LC50 VALUES AND ASSOCIATED 95% CONFIDENCE LIMITS OF JUVENILE SHEEPSHEAD MINNOWS.

Type of Test	Date[a]	96-h LC50 (95% Confidence Limits) (Percent Groundwater by volume)
Untreated	4/26/91 5/01/91	41.3 (35.5 - 47.8)
Chemical Precipitation	4/26/91 5/01/91	No mortality in 100% effluent
UV Oxidation (Test No. 1)	4/26/91 5/01/91	No mortality in 100% effluent
UV Oxidation (Test No. 2)	4/27/91 5/03/91	<5% mortality in 100% effluent
Air Stripping	5/01/91 5/06/91	No mortality in 100% effluent
Carbon Treatment	5/01/91 5/06/91	No mortality in 100% effluent

[a] The upper date for each sample is the date the sample was received at JHU/APL; the lower date is the date the bioassay was completed at JHU/APL.

TABLE 11. SUMMARY OF THE OLD O-FIELD GROUNDWATER BENCH SCALE 48-H MORTALITY DATA OF JUVENILE FATHEAD MINNOWS AND DAPHNIDS - UNTREATED GROUNDWATER (TEST NO. 1).

Treatment (Percent Material by Vol.)	Daphnids (# Dead/# Tested)		Fathead Minnows (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		1/09/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	1/10	0/10	0/10	0/10
32	5/10	1/10	2/10	1/10
56	10/10	10/10	4/10	5/10
100	10/10	10/10	10/10	10/10
100 - pH adjusted to 7 with NaOH	8/10	9/10	3/10	4/10
100 - pH adjusted to 7 with Na_2CO_3	7/10	8/10	5/10	5/10

TABLE 11. (CONTINUED) - UNTREATED GROUNDWATER (TEST NO. 2).

Treatment (Percent Material by Vol.)	Daphnids (# Dead/# Tested)		Fathead Minnows (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		3/21/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	1/10	0/10	0/10
56	3/10	5/10	5/10	4/10
100	10/10	10/10	10/10	10/10
100 - pH adjusted to 7 with Na_2CO_3	6/10	4/10	3/10	3/10

TABLE 11. (CONTINUED) - METALS PRECIPITATION (TEST NO. 1).

Treatment (Percent Material by Vol.)	Daphnids (# Dead/# Tested)		Fathead Minnows (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		1/18/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	0/10	0/10	0/10	0/10
100 - pH adjusted to 7 with HCl	0/10	[a]	1/10	[a]

[a] Only one replicate was run during the test.

TABLE 11. (CONTINUED) - METALS PRECIPITATION (TEST NO. 2).

Treatment (Percent Material by Vol.)	Daphnids (# Dead/# Tested)		Fathead Minnows (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		3/21/21		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	10/10	10/10	10/10	10/10
100 - pH adjusted to 7 with HCl	6/10	4/10	0/10	0/10

TABLE 11. (CONTINUED) - UV OXIDATION (TEST NO. 1).

Treatment (Percent Material by Vol.)	Daphnids (# Dead/# Tested)		Fathead Minnows (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		1/18/91		
0.0	0/10	0/10	0/10	0/10
10	10/10	10/10	2/10	3/10
18	10/10	10/10	10/10	10/10
32	10/10	10/10	10/10	10/10
56	10/10	10/10	10/10	10/10
100	10/10	10/10	10/10	10/10
100 - pH adjusted to 7 with Na_2CO_3	10/10	10/10	10/10	10/10

TABLE 11. (CONTINUED) - UV OXIDATION (TEST NO. 2).

Treatment (Percent Material by Vol.)	Daphnids (# Dead/# Tested)		Fathead Minnows (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		3/23/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	0/10	0/10	0/10	0/10

TABLE 11. (CONTINUED) - UV OXIDATION OF JHU/APL DILUENT WATER.

Treatment (Percent Material by Vol.)	Daphnids (# Dead/# Tested)		Fathead Minnows (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		3/23/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	0/10	0/10	0/10	0/10

TABLE 11. (CONTINUED) - CARBON ADSORPTION.

Treatment (Percent Material by Vol.)	Daphnids (# Dead/# Tested)		Fathead Minnows (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		1/16/91		
0.0	1/10	0/10	0/10	0/10
10	1/10	1/10	0/10	0/10
18	2/10	3/10	0/10	0/10
32	3/10	3/10	0/10	0/10
56	2/10	3/10	0/10	0/10
100	3/10	3/10	0/10	0/10

TABLE 11. (CONTINUED) - CARBON ADSORPTION/BIOLOGICAL SLUDGE.

Treatment (Percent Material by Vol.)	Daphnids (# Dead/# Tested)		Fathead Minnows (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		1/23/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	1/10	0/10	0/10	0/10
100	1/10	2/10	0/10	0/10

TABLE 12. SUMMARY OF THE WATER QUALITY DURING THE OLD O-FIELD GROUNDWATER BENCH SCALE TOXICITY TESTS - UNTREATED GROUNDWATER (TEST NO. 1).

Treatment	0 H New	24 H Old	24 H New	48 H Old	Mean
		pH			
Control	7.29	7.11	7.51	7.38	7.32
10%	7.07	6.73	7.17	7.02	7.00
18%	6.94	6.56	7.06	7.08	6.91
32%	6.57	6.51	6.88	6.93	6.72
56%	6.25	6.34	6.40	6.73	6.43
100%	5.82	5.96	5.84	[a]	5.87
NaOH - pH 7 100%	6.88	6.65	7.07	6.87	6.87
Na_2CO_3 - pH 7 100%	7.04	7.09	7.06	6.93	7.03
		Dissolved Oxygen (mg/L)			
Control	8.5	7.8	8.3	6.4	7.8
10%	8.3	7.0	8.2	6.4	7.5
18%	8.1	6.7	8.3	6.6	7.4
32%	8.3	6.6	8.2	6.0	7.3
56%	8.2	7.3	8.1	5.9	7.4
100%	8.2	8.3	6.6	[a]	7.7
NaOH - pH 7 100%	6.9	6.7	6.3	6.5	6.6
Na_2CO_3 - pH 7 100%	4.2	5.9	6.1	6.5	5.7
		Hardness (mg/L as $CaCo_3$)			
Control	174	170	166	170	170
100%	280	260	264	260	266
		Alkalinity (mg/L as $CaCo_3$)			
Control	80	100	90	80	88
100%	280	260	270	260	268
		Conductivity (μmhos/cm)			
Control	300	280	310	270	290
100%	>500	>500	>500	>500	>500

[a] Data not taken because 100% mortality occurred.

TABLE 12. (CONTINUED) - UNTREATED GROUNDWATER (TEST NO. 2).

Treatment	0 H New	24 H Old	24 H New	48 H Old	Mean
		pH			
Control	8.05	7.58	8.18	7.61	7.86
10%	7.56	7.32	7.07	6.98	7.23
18%	7.18	7.34	6.90	6.98	7.10
32%	6.73	7.14	6.66	7.26	6.95
56%	5.65	6.17	6.39	7.01	6.31
100%	5.65	6.17	[a]	[a]	5.91
Na_2CO_3 - pH 7 100%	7.01	6.39	7.14	6.27	6.70
		Dissolved Oxygen (mg/L)			
Control	8.6	7.2	8.8	8.0	8.2
10%	7.9	6.9	7.7	7.0	7.4
18%	7.9	5.9	7.6	6.9	7.1
32%	7.0	5.9	7.2	6.7	6.7
56%	6.8	5.7	7.0	6.6	6.5
100%	6.0	5.1	[a]	[a]	5.6
Na_2CO_3 - pH 7 100%	6.1	5.5	5.9	5.6	5.8
		Hardness (mg/L as $CaCo_3$)			
Control	176	190	170	180	179
100%	[b]	[b]	[b]	[b]	[b]
		Alkalinity (mg/L as $CaCo_3$)			
Control	80	125	100	130	109
100%	32	10	[a]	[a]	21
		Conductivity (µmhos/cm)			
Control	330	370	330	380	353
100%	680	600	[a]	[a]	640

[a] Data not taken because 100% mortality occurred.
[b] Hardness could be determined because of excessive color in raw groundwater sample.

TABLE 12. (CONTINUED) - METALS PRECIPITATION (TEST NO. 1).

Treatment	0 H New	24 H Old	24 H New	48 H Old	Mean
		pH			
Control	7.78	7.61	7.81	7.87	7.77
10%	8.19	7.83	8.20	7.80	8.01
18%	8.36	7.90	8.36	7.82	8.11
32%	8.62	7.88	8.44	7.75	8.17
56%	8.82	7.98	8.52	7.46	8.20
100%	10.13	8.83	10.01	8.28	9.31
HCl - pH 7					
100%	7.02	7.75	7.04	7.68	7.37
		Dissolved Oxygen (mg/L)			
Control	9.1	8.1	9.0	8.2	8.6
10%	9.0	8.2	8.4	7.8	8.4
18%	8.7	8.1	8.3	7.8	8.2
32%	8.8	8.2	8.3	7.9	8.3
56%	9.0	8.0	8.3	7.9	8.3
100%	9.0	7.8	8.2	7.6	8.2
HCl - pH 7					
100%	8.0	5.9	7.7	6.0	6.9
		Hardness (mg/L as $CaCo_3$)			
Control	176	170	180	160	172
100%	260	240	268	250	255
		Alkalinity (mg/L as $CaCo_3$)			
Control	80	100	70	80	83
100%	25	30	25	25	28
		Conductivity (µmhos/cm)			
Control	310	300	320	300	308
100%	>500	>500	>500	>500	>500

TABLE 12. (CONTINUED) - METALS PRECIPITATION (TEST NO. 2).

Treatment	0 H New	24 H Old	24 H New	48 H Old	Mean
		pH			
Control	8.05	7.58	8.18	7.91	7.93
10%	8.43	8.02	8.63	8.26	8.34
18%	8.81	7.94	9.13	8.30	8.55
32%	9.11	7.83	9.32	8.11	8.59
56%	9.42	7.77	10.01	8.26	8.87
100%	10.87	9.52	[a]	[a]	10.20
HCl - pH 7					
100%	7.11	7.58	7.14	7.99	7.46
		Dissolved Oxygen (mg/L)			
Control	8.6	7.8	8.8	7.6	8.2
10%	7.6	7.0	8.2	7 0	7.5
18%	7.7	7.2	7.8	7.1	7.5
32%	8.0	7.2	8.0	7.1	7.6
56%	7.9	6.2	7.2	6.4	6.9
100%	6.0	7.2	[a]	[a]	6.6
HCl - pH 7					
100%	6.1	5.8	[a]	[a]	6.0
		Hardness (mg/L as $CaCo_3$)			
Control	176	190	170	180	179
100%	194	260	[a]	[a]	227
		Alkalinity (mg/L as $CaCo_3$)			
Control	80	125	100	130	109
100%	100	75	[a]	[a]	88
		Conductivity (µmhos/cm)			
Control	330	370	330	380	353
100%	650	570	[a]	[a]	610

[a] Data not taken because 100% mortality occurred.

TABLE 12. (CONTINUED) - UV OXIDATION (TEST NO. 1).

Treatment	0 H New	24 H Old	24 H New	48 H Old	Mean
		pH			
Control	7.78	7.70	7.81	7.89	7.79
10%	7.50	7.60	7.47	7.75	7.58
18%	7.30	7.49	[a]	[a]	7.40
32%	7.11	7.55	[a]	[a]	7.33
56%	6.38	6.92	[a]	[a]	6.65
100%	4.10	3.58	[a]	[a]	3.84
Na_2CO_3 - pH 7 100%	6.98	6.97	[a]	[a]	6.98
		Dissolved Oxygen (mg/L)			
Control	8.7	8.6	9.0	8.8	8.8
10%	8.7	9.4	9.2	9.0	9.1
18%	8.6	10.0	[a]	[a]	9.3
32%	10.1	12.3	[a]	[a]	11.2
56%	10.8	12.0	[a]	[a]	11.4
100%	11.0	12.1	[a]	[a]	11.6
Na_2CO_3 - pH 7 100%	8.6	6.7	[a]	[a]	7.7
		Hardness (mg/L as $CaCo_3$)			
Control	176	170	180	160	172
100%	352	360	[a]	[a]	356
		Alkalinity (mg/L as $CaCo_3$)			
Control	80 [b]	100 [b]	70 [b]	80 [b]	83 [b]
100%					
		Conductivity (µmhos/cm)			
Control	310	290	320	300	305
100%	>500	>500	[a]	[a]	>500

[a] Data not taken because 100% mortality occurred.
[b] Alkalinity could not be determined because of interference during the titration.

TABLE 12. (CONTINUED) - UV OXIDATION (TEST NO. 2).

Treatment	0 H New	24 H Old	24 H New	48 H Old	Mean
		pH			
Control	7.87	7.95	8.01	7.58	7.85
10%	7.98	7.80	7.99	7.83	7.90
18%	7.96	7.89	7.91	7.84	7.90
32%	7.86	7.80	7.92	7.85	7.86
56%	7.74	7.66	7.72	7.79	7.73
100%	7.45	7.47	7.59	7.68	7.55
		Dissolved Oxygen (mg/L)			
Control	8.0	7.2	7.1	6.2	7.1
10%	8.0	7.2	7.7	6.0	7.2
18%	8.2	6.9	7.7	6.1	7.2
32%	8.5	5.7	7.6	6.3	7.0
56%	9.0	6.6	8.3	6.3	7.6
100%	9.0	7.8	8.0	6.2	7.8
		Hardness (mg/L as $CaCo_3$)			
Control	164	176	160	188	172
100%	380	460	376	432	412
		Alkalinity (mg/L as $CaCo_3$)			
Control	80	100	90	130	100
100%	90	100	90	120	100
		Conductivity (μmhos/cm)			
Control	320	340	315	335	328
100%	610	640	600	700	638

TABLE 12. (CONTINUED) - UV OXIDATION OF JHU/APL DILUENT WATER.

Treatment	0 H New	24 H Old	24 H New	48 H Old	Mean
		pH			
Control	7.87	7.95	8.01	7.58	7.85
10%	8.03	7.83	8.06	7.64	7.89
18%	8.00	7.83	8.03	7.66	7.88
32%	7.94	7.86	8.00	7.68	7.87
56%	7.79	7.79	7.89	7.70	7.79
100%	7.63	7.82	7.76	7.71	7.73
		Dissolved Oxygen (mg/L)			
Control	8.0	7.2	7.1	6.2	7.1
10%	8.1	6.9	7.7	5.9	7.2
18%	8.3	6.0	7.6	6.0	7.0
32%	8.6	6.0	7.7	7.2	7.4
56%	9.0	6.6	7.9	6.2	7.4
100%	9.0	7.8	8.1	6.3	7.8
		Hardness (mg/L as $CaCo_3$)			
Control	164	176	160	188	172
100%	148	158	140	208	164
		Alkalinity (mg/L as $CaCo_3$)			
Control	80	100	90	130	100
100%	135	125	140	150	138
		Conductivity (μmhos/cm)			
Control	320	340	315	335	328
100%	340	410	350	346	362

TABLE 12. (CONTINUED) - CARBON ADSORPTION.

Treatment	0 H New	24 H Old	24 H New	48 H Old	Mean
		pH			
Control	7.47	7.14	7.42	7.21	7.31
10%	7.27	7.05	7.17	7.10	7.15
18%	7.25	7.05	7.10	7.15	7.14
32%	7.15	7.03	6.89	7.05	7.03
56%	7.32	6.99	7.03	6.98	7.08
100%	7.34	7.06	7.00	6.87	7.07
		Dissolved Oxygen (mg/L)			
Control	8.5	8.0	8.7	8.5	8.4
10%	8.5	6.5	8.5	7.7	7.8
18%	8.6	6.8	8.5	7.3	7.8
32%	8.3	6.6	8.6	7.4	7.7
56%	8.3	6.0	8.5	7.0	7.5
100%	8.2	6.2	8.5	7.3	7.6
		Hardness (mg/L as $CaCo_3$)			
Control	156	150	160	146	153
100%	280	260	280	250	268
		Alkalinity (mg/L as $CaCo_3$)			
Control	180	160	170	160	168
100%	90	100	100	110	100
		Conductivity (μmhos/cm)			
Control	330	320	340	330	330
100%	>500	>500	>500	>500	>500

TABLE 12. (CONTINUED) - CARBON ADSORPTION/BIOLOGICAL SLUDGE.

Treatment	0 H New	24 H Old	24 H New	48 H Old	Mean
		pH			
Control	7.90	7.84	8.13	7.77	7.91
10%	8.05	7.91	8.17	7.67	7.95
18%	8.01	7.73	8.18	7.71	7.91
32%	7.99	7.71	8.13	7.54	7.84
56%	7.89	7.60	8.02	7.30	7.70
100%	7.46	7.10	7.84	6.81	7.30
		Dissolved Oxygen (mg/L)			
Control	8.1	7.7	8.8	7.9	8.1
10%	8.2	8.0	8.1	7.6	8.0
18%	8.3	7.5	8.3	7.6	7.9
32%	8.4	7.2	8.0	7.5	7.8
56%	8.3	7.1	8.1	6.7	7.6
100%	8.6	7.2	7.9	7.0	7.7
		Hardness (mg/L as $CaCo_3$)			
Control	194	180	196	192	191
100%	425	400	415	300	385
		Alkalinity (mg/L as $CaCo_3$)			
Control	100	110	95	105	103
100%	15	25	20	10	18
		Conductivity (μmhos/cm)			
Control	300	320	310	325	314
100%	>500	>500	>500	>500	>500

TABLE 13. SUMMARY OF THE OLD O-FIELD GROUNDWATER PILOT SCALE 96-H MORTALITY DATA OF NEONATE DAPHNIDS AND JUVENILE FATHEAD MINNOWS - UNTREATED GROUNDWATER.

Treatment (Percent Material by Vol.)	Daphnid (# Dead/# Tested)		Fathead minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		4/27/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	4/10	4/10	0/10	0/10
56	10/10	10/10	10/10	10/10
100	10/10	10/10	10/10	10/10

TABLE 13. (CONTINUED) - CHEMICAL PRECIPITATION.

Treatment (Percent Material by Vol.)	Daphnid (# Dead/# Tested)		Fathead minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		4/27/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	0/10	0/10	0/10	0/10

TABLE 13. (CONTINUED) - UV OXIDATION (TEST NO. 1).

Treatment (Percent Material by Vol.)	Daphnid (# Dead/# Tested)		Fathead minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		4/27/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	0/10	0/10	0/10	0/10

TABLE 13. (CONTINUED) - UV OXIDATION (TEST NO. 2).

Treatment (Percent Material by Vol.)	Daphnid (# Dead/# Tested)		Fathead minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		4/29/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	1/10	0/10	1/10	0/10
100	0/10	0/10	2/10	0/10

TABLE 13. (CONTINUED) - AIR STRIPPING.

Treatment (Percent Material by Vol.)	Daphnid (# Dead/# Tested)		Fathead minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		5/02/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	0/10	0/10	0/10	0/10

TABLE 13. (CONTINUED) - CARBON.

Treatment (Percent Material by Vol.)	Daphnid (# Dead/# Tested)		Fathead minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		5/02/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	0/10	0/10	0/10	0/10

TABLE 14. SUMMARY OF THE OLD O-FIELD GROUNDWATER PILOT SCALE 96-H MORTALITY DATA OF JUVENILE MYSIDS AND JUVENILE SHEEPSHEAD MINNOWS - UNTREATED GROUNDWATER.

Treatment (Percent Material by Vol.)	Mysid (# Dead/# Tested)		Sheepshead Minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		4/27/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	1/10	1/10	2/10	2/10
56	10/10	10/10	9/10	8/10
100	10/10	10/10	10/10	10/10

TABLE 14. (CONTINUED) - CHEMICAL PRECIPITATION.

Treatment (Percent Material by Vol.)	Mysid (# Dead/# Tested)		Sheepshead Minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		4/27/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	1/10	0/10
100	0/10	0/10	0/10	0/10

TABLE 14. (CONTINUED) - UV OXIDATION (TEST NO. 1).

Treatment (Percent Material by Vol.)	Mysid (# Dead/# Tested)		Sheepshead Minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		4/27/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	0/10	0/10	0/10	0/10

TABLE 14. (CONTINUED) - UV OXIDATION (TEST NO. 2).

Treatment (Percent Material by Vol.)	Mysid (# Dead/# Tested)		Sheepshead Minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		4/29/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	2/10	0/10	0/10	1/10
56	0/10	0/10	2/10	0/10
100	0/10	0/10	1/10	0/10

TABLE 14. (CONTINUED) - AIR STRIPPING.

Treatment (Percent Material by Vol.)	Mysid (# Dead/# Tested)		Sheepshead Minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		5/02/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	0/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	1/10
100	0/10	0/10	0/10	0/10

TABLE 14. (CONTINUED) - CARBON.

Treatment (Percent Material by Vol.)	Mysid (# Dead/# Tested)		Sheepshead Minnow (# Dead/# Tested)	
	Rep 1	Rep 2	Rep 1	Rep 2
		5/02/91		
0.0	0/10	0/10	0/10	0/10
10	0/10	0/10	0/10	0/10
18	0/10	1/10	0/10	0/10
32	0/10	0/10	0/10	0/10
56	0/10	0/10	0/10	0/10
100	0/10	0/10	0/10	0/10

TABLE 15. SUMMARY OF THE WATER QUALITY DURING THE OLD O-FIELD GROUNDWATER PILOT SCALE TOXICITY TESTS - UNTREATED GROUNDWATER (FRESHWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	7.82	7.92	7.67	8.32		7.93
		7.51	7.43	8.09	8.04	7.77
10%	7.02	7.06	6.95	7.44		7.12
		7.11	6.80	7.57	8.01	7.37
18%	6.63	6.71	6.83	7.64		6.95
		6.74	6.86	7.47	8.24	7.33
32%	6.31	6.40	6.42	7.70		6.71
		6.48	6.88	7.25	8.06	7.17
56%	5.96	6.01	6.04	[a]	[a]	6.00
		6.10	5.23	[a]	[a]	5.67
100%	3.23	[a]	[a]	[a]	[a]	3.23
			Dissolved Oxygen (mg/L)			
Control	8.0	7.7	7.4	8.2		7.8
		7.1	7.0	6.2	6.1	6.6
10%	7.4	7.1	7.3	7.2		7.3
		6.4	5.8	5.8	5.9	6.0
18%	7.3	7.0	7.2	7.0		7.1
		6.1	5.7	5.9	6.1	6.0
32%	7.0	6.8	7.0	7.0		7.0
		6.0	5.6	5.3	5.0	5.5
56%	6.8	6.4	6.5	[a]	[a]	6.6
		5.8	6.3	[a]	[a]	6.1
100%	6.0	[a]	[a]	[a]	[a]	6.0

[a] Data not taken because 100% mortality occurred.

TABLE 15. (CONTINUED) - UNTREATED GROUNDWATER (SALTWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	7.78	7.88	7.86	8.12		7.91
		7.39	7.59	8.01	7.94	7.73
10%	6.94	6.98	6.95	7.23		7.02
		7.13	7.22	7.46	7.32	7.28
18%	6.78	6.91	6.92	6.93		6.89
		6.97	7.29	7.19	7.42	7.22
32%	6.63	6.74	6.84	6.86		6.77
		6.77	7.21	7.18	7.43	7.15
56%	6.48	6.51	6.69	6.88		6.56
		6.54	6.84	6.90	6.91	6.69
100%	6.16	[a]	[a]	[a]		6.16
			Dissolved Oxygen (mg/L)			
Control	7.0	7.1	7.2	7.5		7.2
		6.0	6.2	6.9	6.3	6.4
10%	6.7	7.2	6.4	6.5		6.7
		5.9	5.2	5.9	5.8	5.7
18%	6.3	6.5	6.0	6.3		6.3
		5.3	5.3	5.2	5.0	5.2
32%	7.0	6.6	5.5	6.4		6.4
		6.0	5.7	5.1	4.9	5.4
56%	6.6	5.8	5.3	6.4		5.9
		5.7	5.9	5.3	5.0	5.8
100%	6.0	[a]	[a]	[a]		6.0
			Salinity (ppt)			
Control	19.5	20.5	20.0	20.0	20.0	20.0
100%	19.5	20.0	19.0	19.0	19.0	19.3

[a] Data not taken because 100% mortality occurred.

TABLE 15. (CONTINUED) - CHEMICAL PRECIPITATION (FRESHWATER ORGANISMS).

Treatment	0 H New	24 H New	48 H New	72 H New	96 H	Mean
		Old	Old	Old	Old	
			pH			
Control	7.82	7.92	7.67	8.32		7.93
		7.51	7.43	8.09	8.12	7.79
10%	7.47	7.62	7.44	8.44		7.74
		7.92	7.71	7.74	8.46	7.96
18%	7.76	7.91	7.79	8.60		8.01
		7.95	7.77	7.82	8.39	7.98
32%	7.89	7.97	7.80	8.40		8.01
		7.88	7.65	7.76	8.31	7.90
56%	7.76	7.90	7.62	8.17		7.86
		7.78	7.52	7.60	8.11	7.75
100%	7.58	7.75	7.33	7.57		7.56
		7.61	7.30	7.36	7.82	7.52
			Dissolved Oxygen (mg/L)			
Control	8.0	7.7	7.4	8.2		7.8
		7.1	7.0	6.2	6.0	6.6
10%	8.0	8.1	7.7	8.0		8.0
		6.0	6.7	6.0	5.8	6.1
18%	8.1	7.8	7.9	8.0.		8.0
		6.5	6.9	6.3	5.9	6.4
32%	8.0	7.8	7.9	8.1		8.0
		6.6	7.1	6.6	6.1	6.6
56%	7.9	7.9	7.7	8.0		7.9
		6.7	6.7	6.8	6.6	6.7
100%	7.8	7.7	7.9	8.1		7.9
		6.9	6.0	6.5	6.5	6.5

TABLE 15. (CONTINUED) - CHEMICAL PRECIPITATION (SALTWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	7.78	7.88	7.86	8.12		7.91
		7.39	7.50	8.01	8.12	7.76
10%	7.52	7.76	7.73	7.75		7.69
		7.61	7.92	7.88	7.40	7.70
18%	7.65	7.94	8.21	8.12		7.98
		7.50	7.81	7.72	7.78	7.70
32%	7.81	7.96	8.33	8.20		8.08
		7.49	7.76	7.70	7.75	7.68
56%	7.92	8.01	8.47	8.30		8.18
		7.45	7.70	7.62	7.75	7.63
100%	7.93	7.97	8.72	8.52		8.29
		7.48	7.66	7.58	7.67	7.60
			Dissolved Oxygen (mg/L)			
Control	7.0	7.1	7.2	7.5		7.2
		6.0	6.2	6.9	6.3	6.4
10%	7.4	7.5	7.1	7.3		7.3
		6.0	5.1	5.2	4.8	5.3
18%	7.5	7.3	7.1	7.1.		7.3
		5.8	4.9	5.4	4.9	5.3
32%	6.8	7.3	7.0	7.0		7.0
		5.4	4.8	5.3	5.0	5.1
56%	6.9	7.2	7.2	7.1		7.1
		5.6	4.8	5.3	4.9	5.2
100%	6.8	7.1	7.0	6.9		7.0
		5.9	5.0	5.1	5.0	5.3
			Salinity (ppt)			
Control	20.0	20.1	20.0	20.0	20.0	20.0
100%	19.0	19.5	20.0	20.5	21.0	20.0

TABLE 15. (CONTINUED) - UV OXIDATION TEST NO. 1 (FRESHWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	7.82	7.92	7.67	8.32		7.93
		7.51	7.43	8.09	8.02	7.76
10%	7.67	7.48	8.70	7.74		7.90
		7.58	7.94	7.52	7.03	7.52
18%	7.93	7.67	8.85	7.80		8.06
		7.62	7.92	7.61	7.15	7.58
32%	7.90	7.60	8.55	7.67		7.93
		7.57	7.87	7.55	7.09	7.52
56%	7.83	7.56	8.29	7.47		7.79
		7.48	7.74	7.45	7.03	7.43
100%	7.49	7.30	7.69	7.13		7.40
		7.26	7.45	7.09	6.86	7.17
			Dissolved Oxygen (mg/L)			
Control	8.0	7.7	7.4	8.2		7.8
		7.1	7.0	6.2	6.1	6.6
10%	7.7	7.5	7.7	8.1		7.8
		6.5	6.0	6.0	6.3	6.2
18%	7.4	7.6	7.4	8.0		7.6
		6.6	5.9	6.2	6.0	6.2
32%	7.6	7.9	7.3	8.0		7.7
		6.7	6.0	6.1	5.9	6.2
56%	7.8	7.8	7.6	7.9		7.8
		6.5	6.1	6.1	5.8	6.1
100%	7.2	7.7	7.9	7.7		7.6
		6.6	6.3	6.2	5.5	6.2

TABLE 15. (CONTINUED) - UV OXIDATION TEST NO. 1 (SALTWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	7.78	7.88	7.86	8.12		7.91
		7.39	7.59	8.01	8.15	7.79
10%	7.35	7.52	7.60	7.56		7.51
		7.66	7.74	7.73	7.23	7.59
18%	7.69	7.82	8.13	7.96		7.90
		7.52	7.69	7.63	7.55	7.60
32%	7.74	7.94	8.18	8.03		7.97
		7.50	7.64	7.58	7.62	7.59
56%	7.78	7.96	8.16	8.02		7.98
		7.53	7.65	7.57	7.59	7.59
100%	7.79	7.94	8.11	7.91		7.94
		7.58	7.61	7.54	7.56	7.57
			Dissolved Oxygen (mg/L)			
Control	7.0	7.1	7.2	7.5		7.2
		6.0	6.2	6.9	6.4	6.4
10%	7.8	7.6	7.1	7.5		7.5
		5.8	5.1	5.1	4.6	5.2
18%	7.5	7.3	7.2	7.4		7.4
		5.7	4.9	5.2	4.6	5.1
32%	7.3	7.3	7.3	7.3		7.3
		5.8	4.7	4.9	4.5	5.0
56%	7.8	7.6	7.6	7.5		7.6
		5.9	5.2	4.8	4.5	5.1
100%	8.0	7.8	7.8	7.7		7.8
		6.1	5.9	5.0	4.7	5.4
			Salinity (ppt)			
Control	19.5	20.5	20.0	20.0	20.0	20.0
100%	20.5	20.0	21.0	20.0	21.0	20.5

TABLE 15. (CONTINUED) - UV OXIDATION TEST NO. 2 (FRESHWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	7.67	8.32	8.22	8.28		8.12
		8.09	8.11	8.11	7.85	8.04
10%	7.40	8.65	7.53	8.12		7.93
		8.01	7.90	7.94	7.94	7.95
18%	7.67	8.50	7.60	8.56		8.08
		7.97	7.79	7.86	7.80	7.86
32%	7.67	8.41	7.74	8.26		8.02
		7.84	7.66	7.79	7.67	7.74
56%	7.55	8.19	7.69	8.30		7.93
		7.71	7.52	7.66	7.55	7.61
100%	7.20	7.84	7.50	7.87		7.60
		7.47	7.39	7.47	7.05	7.35
			Dissolved Oxygen (mg/L)			
Control	7.4	8.2	7.5	7.7		7.7
		6.2	6.1	6.2	5.9	6.1
10%	8.0	8.2	8.1	7.9		8.1
		6.2	5.2	5.4	4.5	5.3
18%	8.2	8.3	7.9	7.7		8.0
		6.2	5.3	5.0	4.6	5.3
32%	8.1	8.3	7.9	7.7		8.0
		5.9	5.4	5.2	4.6	5.3
56%	8.0	8.3	7.8	7.6		7.9
		5.7	5.3	5.1	4.7	5.2
100%	8.2	8.4	8.0	7.9		8.1
		6.3	6.1	6.0	5.0	5.9

TABLE 15. (CONTINUED) - UV OXIDATION TEST NO. 2 (SALTWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	7.86	8.12	8.31	8.19		8.12
		8.01	8.07	7.99	7.35	7.86
10%	7.82	7.77	8.13	8.14		7.97
		7.84	7.83	7.75	7.74	7.79
18%	8.05	7.95	8.34	8.38		8.18
		7.73	7.71	7.61	7.66	7.68
32%	8.13	8.03	8.47	8.47		8.28
		7.65	7.64	7.49	7.60	7.60
56%	8.14	8.08	8.62	8.60		8.36
		7.60	7.60	7.76	7.51	7.62
100%	8.16	8.07	8.07	8.87		8.29
		7.54	7.54	7.91	7.43	7.61
			Dissolved Oxygen (mg/L)			
Control	7.2	7.5	7.4	7.7		7.5
		6.9	6.8	6.3	5.9	6.5
10%	7.6	7.5	7.4	7.5		7.5
		5.9	5.1	5.0	6.0	5.5
18%	7.4	7.2	7.3	7.4		7.3
		5.7	4.9	4.7	5.5	5.2
32%	7.3	7.2	7.6	7.5		7.4
		4.9	4.4	4.6	5.8	4.9
56%	7.4	7.3	7.9	7.7		7.6
		4.5	4.1	4.0	5.7	4.6
100%	7.3	7.2	8.3	7.9		7.7
		4.8	4.1	4.2	5.9	4.8
			Salinity (ppt)			
Control	20.0	20.0	20.0	20.0	20.0	20.0
100%	21.0	20.0	21.0	21.0	21.0	20.8

TABLE 15. (CONTINUED) - AIR STRIPPING (FRESHWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	8.19	8.38	8.43	8.36		8.34
		8.02	8.09	8.20	8.19	8.13
10%	9.17	8.18	8.55	8.61		8.63
		8.27	8.01	8.01	7.50	7.95
18%	8.93	8.57	8.61	8.62		8.68
		8.09	8.01	8.03	7.65	7.95
32%	8.68	8.45	8.30	8.52		8.49
		7.76	7.75	7.86	7.59	7.74
56%	8.54	8.18	8.21	8.36		8.32
		7.81	7.90	7.79	7.32	7.71
100%	8.44	7.75	7.69	7.77		7.91
		7.33	7.28	7.16	6.84	7.15
			Dissolved Oxygen (mg/L)			
Control	7.9	8.2	8.0	8.3		8.1
		6.3	6.4	6.0	6.2	6.2
10%	8.1	7.5	7.7	8.2		7.9
		7.0	6.8	6.1	6.1	6.5
18%	7.9	7.4	7.3	8.3.		7.7
		6.9	6.4	6.2	6.1	6.4
32%	7.7	7.3	7.5	8.3		7.7
		6.0	6.1	6.4	6.2	6.2
56%	7.6	7.2	7.4	8.1		7.6
		6.2	6.1	6.4	6.3	6.3
100%	8.1	7.5	7.7	8.2		7.9
		6.4	6.0	6.8	6.6	6.5

TABLE 15. (CONTINUED) - AIR STRIPPING (SALTWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	8.41	8.25	8.40	8.29		8.34
		7.94	8.06	8.12	8.21	8.08
10%	8.28	7.98	8.36	8.17		8.20
		7.79	7.38	7.78	7.51	7.62
18%	8.44	8.26	8.39	8.27		8.34
		7.65	7.41	7.79	7.62	7.62
32%	8.51	8.24	8.48	8.34		8.39
		7.75	7.79	7.70	7.58	7.71
56%	8.64	8.28	8.56	8.49		8.49
		7.58	7.91	7.60	7.53	7.66
100%	8.97	8.33	9.09	8.86		8.81
		7.41	7.69	7.40	7.21	7.43
			Dissolved Oxygen (mg/L)			
Control	7.9	7.7	7.9	8.0		7.9
		6.2	5.9	6.1	6.2	6.1
10%	8.2	7.8	7.9	8.0		8.0
		6.1	6.0	5.9	6.3	6.1
18%	8.3	7.4	7.5	7.9		7.8
		5.0	5.1	5.7	5.9	5.4
32%	7.6	7.4	7.6	7.8		7.6
		5.2	5.3	5.6	5.8	5.5
56%	7.4	7.4	7.7	7.8		7.6
		5.5	5.6	5.5	5.6	5.6
100%	7.4	7.6	7.7	7.7		7.6
		5.1	5.2	5.5	5.4	5.3
			Salinity (ppt)			
Control	20.1	20.0	19.8	19.9	20.1	20.0
100%	20.0	20.0	20.0	19.8	19.9	19.9

TABLE 15. (CONTINUED) - CARBON (FRESHWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	8.29	8.41	8.26	8.35		8.33
		8.35	8.01	7.99	7.87	8.06
10%	8.33	8.08	7.90	8.06		8.09
		8.54	7.38	7.70	8.29	7.98
18%	8.97	9.12	8.02	8.09		8.55
		8.45	7.51	7.63	8.23	7.96
32%	8.90	9.11	8.05	8.16		8.55
		8.43	7.53	7.73	8.09	7.95
56%	8.91	9.20	8.30	8.39		8.70
		8.25	7.37	7.55	7.90	7.77
100%	9.23	9.68	9.40	9.41		9.43
		7.74	7.16	7.18	7.26	7.34
			Dissolved Oxygen (mg/L)			
Control	7.9	8.0	7.8	8.3		8.0
		6.2	6.4	6.8	6.5	6.5
10%	8.1	8.3	8.1	8.2		8.2
		6.1	5.9	6.2	6.0	6.1
18%	8.3	8.2	8.1	8.3		8.2
		5.9	5.8	6.2	6.1	6.0
32%	8.2	8.1	8.3	8.3		8.2
		6.0	5.7	6.8	6.6	6.3
56%	8.2	8.1	8.2	8.3		8.2
		5.8	5.6	7.4	7.0	6.5
100%	8.4	8.1	8.3	8.3		8.3
		6.3	6.1	7.3	7.1	6.7

TABLE 15. (CONTINUED) - CARBON (SALTWATER ORGANISMS).

Treatment	0 H New	24 H New / Old	48 H New / Old	72 H New / Old	96 H Old	Mean
			pH			
Control	8.29	8.43	8.25	8.39		8.34
		7.99	8.11	8.11	8.00	8.05
10%	7.57	8.13	8.15	8.18		8.01
		8.03	7.85	8.17	7.76	7.95
18%	8.08	8.32	8.24	8.04		8.17
		8.07	7.81	8.19	7.73	7.95
32%	8.16	8.36	8.37	8.10		8.25
		8.12	7.68	7.65	7.68	7.78
56%	8.38	8.43	8.54	8.15		8.38
		8.08	7.73	7.56	7.66	7.76
100%	9.03	8.48	9.13	8.26		8.73
		7.88	7.66	7.60	7.45	7.65
			Dissolved Oxygen (mg/L)			
Control	8.3	7.5	7.8	7.9		7.9
		5.9	6.0	6.1	6.2	6.1
10%	8.3	7.8	8.0	7.9		8.0
		6.6	6.2	5.7	6.0	6.1
18%	8.1	8.0	8.1	7.8		8.0
		6.5	6.3	5.4	5.7	6.0
32%	8.2	8.0	7.9	7.8		8.0
		6.4	6.0	5.5	5.2	5.8
56%	8.3	8.1	8.0	7.7		8.0
		6.8	6.4	6.1	5.9	6.3
100%	8.3	8.0	8.1	7.9		8.1
		6.8	6.6	5.9	5.6	6.2
			Salinity (ppt)			
Control	20.0	21.0	20.0	20.0	20.0	20.2
100%	19.0	18.0	20.0	19.0	19.0	19.0

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a CORNING Laboratory Services Company

MUTAGENICITY TEST ON

RAW GROUNDWATER

IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY (AMES TEST) MODIFIED FOR DIRECT WATER SAMPLES

FINAL REPORT

AUTHOR

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PERFORMING LABORATORY

Hazleton Washington, Inc.
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LABORATORY PROJECT ID

HWA Study No.: 14544-0-401W

SUBMITTED TO

Johns Hopkins University
Applied Physics Laboratory
4800 Atwell Road
Shady Side, MD 20764

STUDY COMPLETION DATE

June 10, 1991

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HAZLETON
WASHINGTON

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples

STUDY NO.: 14544-0-401W

PROTOCOL NO.: 401W EDITION NO.: 16

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection - Date	Findings Reported	Auditor
Preparation of S9 Mix - 05/02/91	05/02/91	D. Wallace
Final Report Review - 06/07/91	06/07/91	D. Wallace

Dana J. Wallace 6-7-91
Quality Assurance Unit Date Released

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COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160). To the best of the signer's knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The stability of the test article under the conditions of administration was the responsibility of the Sponsor. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of the HWA Division of Molecular and Cellular Services, 5516 Nicholson Lane, Kensington, Maryland 20895. After two years, these records will be transferred to permanent archives at Hazleton Washington, Vienna, Virginia.

SUBMITTED BY:

Study Director:

Timothy E. Lawlor, M.A.
Microbial Mutagenesis
Department of Molecular and Cellular Services

6·10·91
Study Completion Date

HAZLETON
WASHINGTON

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HAZLETON
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SECTION I. SUMMARY

INTRODUCTION AND CONCLUSIONS

SUMMARY

A. Introduction

At the request of Johns Hopkins University, Hazleton Washington, Inc. examined water sample Raw Groundwater for mutagenic activity in the Salmonella/ Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor-induced rat liver.

The tester strains used in this study were TA98 and TA100. The assay was conducted using two plates per dose level in the presence and absence of microsomal enzymes. Six dose levels of the test article, 3.00, 2.00, 1.00, 0.500, 0.200 and 0.100 ml per plate, were tested in both the presence and absence of S9 along with the appropriate vehicle controls (three plates per dose), negative controls and positive controls.

B. Conclusions

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples indicate that under the conditions of this study, Johns Hopkins University's test article, Raw Groundwater, did not cause a positive increase in the numbers of histidine revertants per plate with tester strains TA98 and TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

SECTION II. STUDY INFORMATION

HAZLETON
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STUDY INFORMATION

A. Sponsor: Johns Hopkins University

B. Test Article: Raw Groundwater

1. Physical Description: cloudy orange liquid

2. Date Received: 04/29/91

C. Type of Assay: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Modified for Direct Water Samples)

1. Protocol Number: HWA Protocol 401W, Edition 16

2. HWA Study Number: 14544-0-401W

D. Study Dates

1. Study Initiation Date: 04/29/91

2. Test Initiation in the Laboratory: 04/30/91

3. Test Completion in the Laboratory: 05/10/91

E. Study Personnel

Study Director: Timothy E. Lawlor, M.A.

Laboratory Supervisor: Michael S. Mecchi, B.S.

Technician: Theodora Brown

Technician: Sow Hoong Hon, B.S.

SECTION III. MATERIALS AND METHODS

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames et al (1975).

A. Media and Reagents

1. Top Agar for Selection of Histidine Revertants: Since different volumes of neat water sample will be added directly to the tubes containing top agar, it is necessary to prepare tubes containing different concentrations of top agar components (agar, NaCl, histidine and biotin), such that once the appropriate volume of water sample has been added, the top agar component concentration in all top agar tubes will be comparable. A series of top agar tubes was prepared as follows:

Top Agar Tube	Top Agar Component	Concentration (%)	ml of 0.5mM Histidine/ Biotin Solution Added per ml of Top Agar	ml of Supplemented Top Agar Added per Tube	ml of Test Article Added per Tube
1.0 X	Agar	0.60			
	NaCl	0.50	0.07	3.0	0.1
1.0 X	Agar	0.60			
	NaCl	0.50	0.07	3.0	0.2
1.5 X	Agar	0.72			
	NaCl	0.60	0.08	2.5	0.5
2.0 X	Agar	0.90			
	NaCl	0.75	0.10	2.0	1.0
3.0 X	Agar	1.80			
	Nacl	1.50	0.20	1.0	2.0
4.0 X	Agar	2.40			
	NaCl	2.00	0.25	1.0	3.0

2. Minimal Bottom Agar: Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (W/V) agar and 0.2% (W/V) glucose.

3. Nutrient Broth: Nutrient Broth used for growing overnight cultures of the tester strains was Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

4. Exogenous Metabolic Activation

a. Liver Microsomal Enzymes - S9 Homogenate: S9 Liver homogenate for use in the mutagenicity assay, prepared as described below, was purchased from Molecular Toxicology, Inc., Annapolis, MD 21401, Batch 0327, 37.8 mg of protein per ml.

1) Species, Strain, Sex, Inducer: Liver microsomal enzymes were prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 (200 mg per ml in corn oil) at 500 mg/kg. Five days after i.p. injection with the Aroclor, the rats were sacrificed by decapitation, and their livers were excised.

2) Homogenate Preparation: The preparation of the microsomal enzyme fraction was carried out with sterile glassware and solutions at 6 ± 4°C. The livers were excised, weighed, and placed in a beaker containing 3 ml of 0.15M KCl per gram of wet liver, and homogenized. The homogenate was centrifuged at 9000 x g for 10 minutes. Small aliquots of the supernatant (referred to by Ames as the S9 fraction) were distributed into freezing ampules which were stored at ≤ -65°C.

3) S9 Characterization: The S9 homogenate was characterized (using the Ames Assay) for its ability to metabolize selected promutagens to their mutagenic forms, as described by deSerres and Shelby (1979).

b. S9 Mix: The S9 mix was prepared immediately before its use in the mutagenicity assay. One ml of the microsomal enzyme reaction mixture (S9 mix) contained the following components:

H_2O	0.70 ml
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 ml
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 ml
0.2M $MgCl_2$/0.825M KCl	0.04 ml
S9 Homogenate	0.10 ml
	1.00 ml

When required, 0.5 ml of the S9 mix was added to the soft agar overlay per plate.

B. Test System

1. Tester Strains

The tester strains used were the Salmonella typhimurium histidine auxotrophs TA98 and TA100 as described by Ames et al (1975).

TESTER STRAIN GENOTYPES

Histidine Mutation		Additional Mutations		
hisG46	hisD3052	LPS	Repair	R Factor
TA100	TA98	rfa	uvrB	+R

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the uvrB gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strain TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototropy) by frameshift mutagens. Tester strain TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

a. Source of Tester Strains: The tester strains in use at HWA were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

b. Storage of the Tester Strains

1) Frozen Permanent Stocks: Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (approximately 0.5 - 1.5 ml) at $\leq$-65°C.

2) Master Plates: Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 μM), biotin (3 μM), and ampicillin (25 μg/ml). Tester strain master plates were stored at 6 $\pm$ 4°C.

c. Preparation of Overnight Cultures: Overnight cultures were prepared by transferring a colony from the appropriate master plate to a flask containing culture medium. In order to assure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 $\pm$ 25 rpm; incubation, 37 $\pm$ 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began. Cultures were harvested once a predetermined turbidity was

reached as determined by a percent transmittance (%T) reading on a spectrophotometer. Overgrowth of cultures can result in their loss of sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached.

d. Confirmation of Tester Strain Genotypes: Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

1) rfa Wall Mutation: The presence of the rfa wall mutation was confirmed by demonstration of sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

2) pKM101 Plasmid R-factor: The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

3) Characteristic Number of Spontaneous Revertants: The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with 3.00 ml of deionized water on selective media.

C. Experimental Design

1. Mutagenicity Assay

The mutagenicity assay was performed using tester strains TA98 and TA100, both in the presence and absence of microsomal enzymes (S9 mix). Six dose levels of the test article, 3.00, 2.00, 1.00, 0.50, 0.20 and 0.10 ml per plate, were tested along with the appropriate vehicle, negative, and positive controls as specified by the New Jersey Department of Environmental Protection. If the test article exhibited toxicity in the assay in two or more doses, the assay would be repeated at lower concentrations.

a. Frequency and Route of Administration: The test system was exposed to the test article via the plate incorporation methodology originally described by Ames et al (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. All dose levels of test article, negative controls, and positive controls were plated in duplicate and the vehicle controls were plated in triplicate.

D. Controls

1. Positive Controls

Combinations of positive controls and tester strains plated concurrently with the assay are listed below.

POSITIVE CONTROL AND TESTER STRAIN COMBINATIONS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 µg
TA98	-	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	-	sodium azide	2.0 µg

a. Source and Grade of Positive Control Articles: 2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade; 2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98%; sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade.

2. Vehicle Controls

Sterilized deionized water (HWA Batch #193) vehicle controls were plated for both tester strains in the presence and absence of S9. The vehicle controls consisted of a 3.0 ml aliquot of deionized water (equal to the maximum aliquot of test article plated), along with an aliquot of the appropriate tester strain and an aliquot of S9 mix (when appropriate), plated on selective agar.

3. Negative Controls

Negative controls were plated for both tester strains in the presence and absence of S9. The negative controls consisted of an aliquot of the appropriate tester strain and S9 mix (when appropriate), plated on selective agar.

4. Sterility Controls

a. S9 Mix Sterility Determination: In order to determine the sterility of the S9 mix, a 0.5 ml aliquot was plated on selective agar.

E. Plating Procedures

The plating procedures employed are similar to those described by Ames et al (1975).

1. Test System Identification

Each plate was labeled with a code system which identified the test article, tester strain, test phase, dose level, and activation condition.

2. Test Article Plating Procedure

The S9 mix was prepared immediately before its use in the experimental procedure. An aliquot of tester strain (100 µl), the appropriate volume of vehicle or neat test article, and 0.5 ml of S9 mix (when necessary) were added to an appropriate amount and concentration of molten selective top agar (See III.A.1.) held in a 13 x 100 mm test tube at 45 ± 2°C. After vortexing, the mixture was overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 hours at 37 ± 2°C.

F. Scoring Plates

Plates which were not scored immediately after the 48 ± 8 hour incubation period were held at 6 ± 4°C until such time that scoring could occur.

1. Colony Counting

Revertant colonies for the negative, vehicle, and test article treated plates were counted by hand. The positive control plates were counted by automated colony counter.

2. Evaluation of the Bacterial Background Lawn

The condition of the background bacterial lawn was evaluated for evidence of test article cytotoxicity and precipitate. The cytotoxicity was scored relative to the vehicle control plate and is noted along with the revertant counts for all plates at that dose level on the data tables using the code system presented at the end of the Materials and Methods Section.

3. Analysis of the Data

For all replicate platings, the mean number of revertants per plate was calculated and the standard deviation around the mean was also calculated. The results of these calculations are presented in tabular form in the Data Tables Section of this report.

G. Criteria for Determination of a Valid Test

The following criteria must be met for the assay to be considered valid:

1. Tester Strain Integrity

a. rfa Wall Mutation: In order to demonstrate the presence of the deep rough mutation, tester strain cultures must exhibit sensitivity to crystal violet.

b. pKM101 Plasmid R-Factor: In order to demonstrate the presence of the pKM101 Plasmid R-factor, tester strains TA98 and TA100 must exhibit resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants: Tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The acceptable ranges are as follows:

TA98	8 - 60
TA100	60 - 240

d. Tester Strain Titers: In order to ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to 5.0×10^8 and/or have reached a target level of turbidity demonstrated to produce cultures with titers greater than or equal to 5.0×10^8.

e. Positive Control Values: All positive controls must exhibit at least a three-fold increase in the number of revertants per plate over the mean value for the vehicle control for the respective strain.

2. Cytotoxicity

a. Acceptable Number of Non-toxic Dose Levels: A minimum of three non-toxic dose levels are required to evaluate assay data.

H. Evaluation of Test Results

1. Tester Strains TA98 and TA100

For a test article to be considered positive, it must cause at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

L. References

Ames, B.N., J. McCann and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Research 31:347-364 (1975).

deSerres, F.J., and M.D. Shelby. Recommendations on Data Production and Analysis using the Salmonella/Microsome Mutagenicity Assay: Mutation Research 64:159-165 (1979).

Maron, D.M., and B. Ames. Revised Methods for the Salmonella Mutagenicity Test. Mutation Research 113:173-215 (1983).

Vogel, H.J., and D.M. Bonner. Acetylornithinase of E. coli: Partial Purification and Some Properties, J. Biol. Chem. 218:97-106 (1956).

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS OF BACKGROUND LAWN
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

SECTION IV. RESULTS AND CONCLUSIONS

RESULTS

A. Test Article Handling

The water sample, Raw Groundwater, was stored refrigerated at 5 ± 3°C until used in the assay. The test article was filter sterilized using a 0.45 µm filter to remove any particulate matter and possible bacterial or other contaminant that would interfere with the assay.

B. Mutagenicity Assay

The dose levels selected for the mutagenicity assay were 3.00, 2.00, 1.00, 0.500, 0.200, and 0.100 ml of unconcentrated test article per plate in the presence and absence of S9, as specified by the New Jersey Department of Environmental Protection. The mutagenicity assay results for Raw Groundwater are presented in Tables 1 and 2. These data were generated in Experiment 14544-B2 (in an initial experiment, 14544-B1, no data was generated due to the malfunction of an incubator). The data are presented as mean revertants per plate ± standard deviation for each treatment and control group (Table 2) and as individual plate counts (Table 1).

In Experiment 14544-B2, all data were acceptable and no positive increases in the number of histidine revertants per plate were observed.

All criteria for a valid study were met.

CONCLUSIONS

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples indicate that under the conditions of this study, Johns Hopkins University's test article, Raw Groundwater, did not cause a positive increase in the number of histidine revertants per plate with tester strains TA98 and TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

SECTION V. DATA TABLES

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TABLE 1

INDIVIDUAL PLATE COUNTS

TEST ARTICLE ID: Raw Groundwater

EXPERIMENT ID: 14544-B2 VEHICLE: deionized water

DATE PLATED: 02-May-91 DATE COUNTED: 09-May-91

DOSE/PLATE		REVERTANTS PER PLATE TA98 1	TA98 2	TA98 3	TA100 1	TA100 2	TA100 3	BACKGROUND LAWN*
MICROSOMES: Rat Liver								
NEGATIVE CONTROL		38	35		97	92		1
VEHICLE CONTROL		34	28	28	116	105	94	1
TEST ARTICLE	0.100 ml	31	31		107	91		1
	0.200 ml	40	40		114	103		1
	0.500 ml	34	33		116	107		1
	1.00 ml	29	25		154	139		1
	2.00 ml	32	25		115	114		1
	3.00 ml	27	20		118	112		1
POSITIVE CONTROL **		1140	1130		1042	959		1
MICROSOMES: None								
NEGATIVE CONTROL		23	13		91	88		1
VEHICLE CONTROL		16	15	14	94	83	72	1
TEST ARTICLE	0.100 ml	29	13		90	79		1
	0.200 ml	32	27		99	93		1
	0.500 ml	22	13		107	91		1
	1.00 ml	27	22		102	86		1
	2.00 ml	16	12		109	108		1
	3.00 ml	24	19		104	95		1
POSITIVE CONTROL ***		148	115		656	658		1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

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WASHINGTON

TABLE 2

SUMMARY OF TEST RESULTS

TEST ARTICLE ID: Raw Groundwater

EXPERIMENT ID: 14544-B2 VEHICLE: deionized water

DATE PLATED: 02-May-91 DATE COUNTED: 09-May-91

	DOSE/PLATE	MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION TA98 MEAN	TA98 S.D.	TA100 MEAN	TA100 S.D.	BACKGROUND LAWN*
MICROSOMES: Rat Liver						
NEGATIVE CONTROL		37	2	95	4	1
VEHICLE CONTROL		30	3	105	11	1
TEST ARTICLE	0.100 ml	31	0	99	11	1
	0.200 ml	40	0	109	8	1
	0.500 ml	34	1	112	6	1
	1.00 ml	27	3	147	11	1
	2.00 ml	29	5	115	1	1
	3.00 ml	24	5	115	4	1
POSITIVE CONTROL **		1135	7	1001	59	1
MICROSOMES: None						
NEGATIVE CONTROL		18	7	90	2	1
VEHICLE CONTROL		15	1	83	11	1
TEST ARTICLE	0.100 ml	21	11	85	8	1
	0.200 ml	30	4	96	4	1
	0.500 ml	18	6	99	11	1
	1.00 ml	25	4	94	11	1
	2.00 ml	14	3	109	1	1
	3.00 ml	22	4	100	6	1
POSITIVE CONTROL ***		132	23	657	1	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
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hp = heavy precipitate (requires hand count)

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a CORNING Laboratory Services Company

MUTAGENICITY TEST ON

AN EXTRACT OF THE WATER SAMPLE

RAW GROUNDWATER

IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY (AMES TEST) MODIFIED FOR XAD-2 RESIN EXTRACTS

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Hazleton Washington, Inc.
5516 Nicholson Lane
Kensington, Maryland 20895

LABORATORY PROJECT ID

HWA Study No.: 14544-1-401X

SUBMITTED TO

Johns Hopkins University
Applied Physics Laboratory
4800 Atwell Road
Shady Side, MD 20764

STUDY COMPLETION DATE

June 12, 1991

United States • Germany • Japan • France • United Kingdom
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WASHINGTON

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts

STUDY NO.: 14544-1-401X

PROTOCOL NO.: 401X EDITION NO.: 17

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection - Date	Findings Reported	Auditor
Preparation of S9 Mix - 05/15/91	05/15/91	D. Wallace
Final Report Review - 06/12/91	06/12/91	P. Postal

Patti Postal 6·12·91

Quality Assurance Unit Date Released

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COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160). To the best of the signer's knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The stability of the test article under the conditions of administration was the responsibility of the Sponsor. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of the HWA Division of Molecular and Cellular Services, 5516 Nicholson Lane, Kensington, Maryland 20895. After two years, these records will be transferred to permanent archives at Hazleton Washington, Vienna, Virginia.

SUBMITTED BY:

Study Director:

Timothy E. Lawlor, M.A.
Microbial Mutagenesis
Department of Molecular and Cellular Toxicology

6·12·91
Study Completion Date

HAZLETON
WASHINGTON

TABLE OF CONTENTS

SECTION L SUMMARY

INTRODUCTION AND CONCLUSIONS

SUMMARY

A. Introduction

At the request of Johns Hopkins University, Hazleton Washington, Inc. examined an extract of the water sample, Raw Groundwater, for mutagenic activity in the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver.

The tester strains used in this study were TA98 and TA100. The assay was conducted using two plates per dose level in the presence and absence of microsomal enzymes. Six dose levels of the test article were tested, from 500 to 12.4 μg per plate in both the presence and absence of S9. Vehicle controls (three plates per dose), negative controls, resin controls and positive controls were plated as part of the assay.

The doses tested in the mutagenicity assay were selected based on the amount of extractable organics recovered from the test article.

B. Conclusions

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts indicate that under the conditions of this study, an extract of Johns Hopkins University's test article, Raw Groundwater, did cause a positive increase in the number of histidine revertants per plate with tester strain TA98 in the presence of S9 in two independent trials (3.6 and 4.5-fold). A positive increase was also observed with tester strain TA100 in the presence of S9 (2.0-fold) in the initial experiment. However, in the confirmatory assay, this positive increase could not be reproduced. No positive increases were observed with either tester strain in the absence of S9.

SECTION II. STUDY INFORMATION

STUDY INFORMATION

A. Sponsor: Johns Hopkins University

B. Test Article: Raw Groundwater

1. Physical Description, Test Article: cloudy orange liquid

2. Date Received: 04/29/91

3. Physical Description, Extract: cloudy brown liquid

C. Type of Assay: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts

1. Protocol Number: HWA Protocol 401X, Edition 17

2. HWA Study Number: 14544-1-401X

D. Study Dates

1. Study Initiation Date: 04/29/91

2. Experimental Start: 05/15/91

3. Experimental Termination: 06/03/91

E. Study Personnel

Study Director: Timothy E. Lawlor, M.A.

Laboratory Supervisor: Michael S. Mecchi, B.S.

Research Assistant: Ralph S. McCrea, B.S.

Technician: Theodora Brown

Technician: Sow Hoong Hon, B.S.

SECTION III. MATERIALS AND METHODS

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames et al (1975).

MATERIALS

A. Tester Strains

The tester strains used were the Salmonella typhimurium histidine auxotrophs TA98 and TA100 as described by Ames et al (1975). The specific genotypes of these strains are shown in Table 1.

TABLE 1. TESTER STRAIN GENOTYPES

Histidine Mutation		Additional Mutations		
hisG46	hisD3052	LPS	Repair	R Factor
TA100	TA98	rfa	uvrB	+R

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the uvrB gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strain TA98 is reverted from histidine dependence (auxctrophy) to histidine independence (prototropy) by frameshift mutagens. Tester strain TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

1. Source of Tester Strains

The tester strains in use at HWA were received directly from Dr. Bruce Ames, Dept. of Biochemistry, University of California, Berkeley.

2. Storage of the Tester Strains

a. Frozen Permanent Stocks

Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (approximately 0.5 - 1.5 ml) at ≤-70°C.

b. Master Plates

Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 μM), biotin (3 μM), and for strains containing the R-factor, ampicillin (25 μg/ml). Tester strain master plates were stored at 5 ± 3°C.

3. Preparation of Overnight Cultures

a. Inoculation

Overnight cultures for use in all testing procedures were inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 ± 25 rpm; incubation, 37 ± 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began.

b. Harvest

To ensure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring of culture turbidity. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance (%T) reading on a spectrophotometer. This target turbidity ensures that cultures have reached a density of at least 0.5 X 10^9 cells per ml and that the cultures have not overgrown. Overgrown (stationary) cultures may exhibit decreased sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached and were placed at 5 ± 3°C.

4. Confirmation of Tester Strain Genotypes

Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

a. rfa Wall Mutation

The presence of the rfa wall mutation was confirmed by demonstration of the cultures sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

b. pKM101 Plasmid R-factor

The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

c. Characteristic Number of Spontaneous Revertants

The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with the appropriate vehicle on selective media.

5. Tester Strain Media

a. Culturing Broth

The broth used to grow overnight cultures of the tester strains was Vogel-Bonner salt solution supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

b. Agar Plates

Bottom agar (25 ml per 15 x 100 mm petri dish) was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (W/V) agar and 0.2% (W/V) glucose.

c. Overlay Agar for Selection of Histidine Revertants

Overlay (top) agar was prepared with 0.7% agar (W/V) and 0.5% NaCl (W/V) and was supplemented with 10 ml of 0.5 mM histidine/biotin solution per 100 ml agar. When S9 mix is required, 2.0 ml of the supplemented top agar is used in the overlay. However, when S9 is not required, water is added to the supplemented top agar (0.5 ml of water per 2 ml of supplemented top agar) and the resulting 2.5 ml of diluted supplemented top agar is used for the overlay. This dilution ensures that the final top agar and amino acid concentrations remain the same both in the presence and absence of S9.

B. Liver Microsomal Enzyme Reaction Mixture (S9 Mix)

1. S9 Homogenate

Liver microsomal enzymes (S9 homogenate) were purchased from Molecular Toxicology, Inc., Annapolis, MD 20401, Batch 0327 (37.8 mg of protein per ml). The homogenate was prepared from male Sprague-Dawley rats that had been injected (i.p.) with Aroclor™ 1254 (200 mg per ml in corn oil) at 500 mg/kg as described by Ames et al, 1975.

2. S9 Mix

The S9 mix was prepared immediately prior to its use in any experimental procedure. The S9 mix contained the components indicated in Table 2.

TABLE 2. S9 MIX COMPONENTS

H_2O	0.70 ml
1M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 ml
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 ml
0.825M KCl/0.2M $MgCl_2$	0.04 ml
S9 Homogenate	0.10 ml
	1.00 ml

C. Controls

1. Vehicle Controls

Dimethylsulfoxide (DMSO, CAS# 67-68-5, Sigma Chemical Co., Lot 30H0608, 99+%) vehicle controls were plated for both tester strains in the presence and absence of S9. The vehicle control was plated, using a 50 µl aliquot of DMSO (equal to the maximum aliquot of test article dilution plated), along with a 100 µl aliquot of the appropriate tester strain and a 500 µl aliquot of S9 (when necessary), on selective agar.

2. Negative Controls

Negative controls were plated for both tester strains in the presence and absence of S9. The negative controls consisted of a 100 µl aliquot of the appropriate tester strain and S9 (when appropriate) plated on selective agar.

3. Resin Controls

Resin controls were plated for both tester strains in the presence and absence of S9, only in the initial assay. A sample of deionized water was extracted and concentrated using the same XAD-2 resin adsorption procedure used for the test article. The resin control was tested at a single concentration. The resin controls consisted of a 50 µl aliquot of the resin control extract, a 100 µl aliquot of tester strain and an aliquot of S9 (when appropriate) plated on selective agar.

4. Positive Controls

The combinations of positive controls, activation condition and tester strains plated concurrently with the assay are indicated in Table 3.

TABLE 3. POSITIVE CONTROLS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 µg
TA98	-	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	-	sodium azide	2.0 µg

a. Source and Grade of Positive Control Articles

2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade; 2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98%; sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade.

5. Sterility Controls

a. S9 Mix

The S9 mix was checked for sterility by plating 0.5 ml on selective agar.

METHODS

A. Mutagenicity Assay

1. Design

The assay was performed using tester strains TA98 and TA100 both in the presence and absence of S9 mix. Six dose levels of the test

article extract were tested along with the appropriate vehicle, negative, resin and positive controls. The dose levels tested were selected based on the amount of extractable organics recovered in the extraction procedure.

2. Frequency and Route of Administration

The tester strains were exposed to the test article via the plate incorporation methodology originally described by Ames et al (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. In the plate incorporation methodology, the test article, the tester strain and the S9 mix (where appropriate) are combined in molten agar which is overlaid onto a minimal agar plate. Following incubation at 37 ± 2°C for 48 ± 8 hr, revertant colonies will be counted. All dose levels of test article, negative controls, resin controls and positive controls were plated in duplicate, and the vehicle controls were plated in triplicate.

B. Plating Procedures

Each plate was labeled with a code which identified the test article, test phase, tester strain, activation condition and dose level. The S9 mix and dilutions of the test article were prepared immediately prior to their use.

When S9 mix was not required, 100 µl of tester strain and 50 µl of vehicle or test article dose was added to 2.5 ml of molten selective top agar (maintained at 45 ± 2°C). When S9 mix was required, 500 µl of S9 mix, 100 µl of tester strain and 50 µl of vehicle or test article dose was added to 2.0 ml of molten selective top agar. After the required components had been added, the mixture was vortexed and overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 hr at 37 ± 2°C. Positive controls and the resin control were plated using a 50 µl plating aliquot.

C. Scoring the Plates

Plates which were not evaluated immediately following the incubation period were held at 5 ± 3°C until such time that colony counting and bacterial background lawn evaluation could take place.

1. Bacterial Background Lawn Evaluation

The condition of the bacterial background lawn was evaluated for evidence of cytotoxicity and test article precipitate. Evidence of cytotoxicity was scored relative to the vehicle control plate and was recorded along with the revertant counts for all plates at that dose level on the data tables using the code system presented at the end of the Materials and Methods Section.

2. Counting Revertant Colonies

The number of revertant colonies per plate for the vehicle controls and all plates containing test article were counted manually. The number of revertant colonies per plate for the positive controls were counted by automated colony counter.

D. Analysis of Data

For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The results of these calculations are presented in tabular form in the Data Tables Section of this report.

EVALUATION OF TEST RESULTS

Before assay data can be evaluated, the criteria for a valid assay must be met.

A. Criteria For A Valid Assay

The following criteria are used to determine a valid assay:

1. Tester Strain Integrity

a. rfa Wall Mutation

To demonstrate the presence of the rfa wall mutation, tester strain cultures must exhibit sensitivity to crystal violet.

b. pKM101 Plasmid

To demonstrate the presence of the R-factor plasmid, pKM101, cultures of tester strains TA98 and TA100 must exhibit resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants

To demonstrate the requirement for histidine, the tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable ranges for the vehicle controls are as follows:

TA98	8 - 60
TA100	60 - 240

d. Tester Strain Culture Density

To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures must be greater than or equal to 0.5×10^9 bacteria per ml and/or have reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 0.5×10^9 bacteria per ml.

e. Positive Control Values

(1) Positive Control Values in the Absence of S9 Mix

To demonstrate that the tester strains are capable of identifying a mutagen, the mean value of a positive control for a respective tester strain must exhibit at least a 3-fold increase over the mean value of the vehicle control for that strain.

(2) Positive Control Values in the Presence of S9 Mix (S9 Mix Integrity)

To demonstrate that the S9 mix is capable of metabolizing a promutagen to its mutagenic form(s), the mean value of the positive control for a respective tester strain in the presence of the S9 mix must exhibit at least a 3-fold increase over the mean value of the vehicle control for that strain.

An acceptable positive control in the presence of S9 for a specific strain will be evaluated as having demonstrated both the integrity of the S9 mix and the ability of the tester strain to detect a mutagen.

2. Cytotoxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data.

B. Criteria For A Positive Response

Once the criteria for a valid assay have been met, responses observed in the assay are evaluated as follows:

1. Tester Strains TA98 and TA100

For a test article to be considered positive, it must produce at least a 2-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

RECORDS TO BE MAINTAINED

All raw data, reports, protocols and modifications will be maintained by the Department of Molecular and Cellular Toxicology of HWA for a period of up to two years following submission of the final report to the Sponsor. After two years, all raw data and reports will be transferred to the HWA archives for permanent storage.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Research 31:347-364 (1975).

Maron, D.M., and Ames, B. Revised Methods for the Salmonella Mutagenicity Test. Mutation Research 113:173-215 (1983).

Vogel, H.J., and D.M. Bonner. Acetylornithinase of E. coli: Partial Purification and Some Properties, J. Biol. Chem. 218:97-106 (1956).

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS OF BACKGROUND LAWN
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

SECTION IV. RESULTS AND CONCLUSIONS

RESULTS

A. Test Article Handling

The water sample, Raw Groundwater, was held at 5 ± 3°C until extracted and concentrated by the XAD-2 resin adsorption as described in the "Environmental Water Sample Processing Protocol No. 12, October, 1989."

The test article (29.2 liters) was passed through XAD-2 resin and solvent exchanged to dimethylsulfoxide (DMSO) using the procedure described below. Deionized water (48 liters) was also processed in the same manner as an internal control for the extraction procedure (resin control).

A chromatographic column (22 mm I.D.) was slurry-packed with 15 g of XAD-2 resin and washed with acetone (CAS# 67-64-1, Fisher Scientific, Lot 900324) and water. All extraction procedures were performed at 5 ± 3°C. The test article was passed through the XAD-2 column using gravity flow at a rate of 60 to 80 drops/min. Material adsorbed to the column was eluted with acetone and methylene chloride (CAS# 75-09-2, Fisher Scientific, Lot 890625). The eluate was reduced in volume using a rotary evaporator. Duplicate aliquots of the concentrate were transferred to tared aluminum weighing dishes, the solvent allowed to evaporate off, and the amount of material extracted from the water samples determined gravimetrically. The remainder of the extract was solvent exchanged into 0.5 ml dimethylsulfoxide (DMSO, CAS# 67-68-5, Sigma Chemical Co., Lot 30H0608, 99+%).

Extractable Organics

	Sample Volume	Extractives mg/0.5 ml
Raw Groundwater	29.2 Liters	13.5
Resin Control (deionized water)	48 Liters	0.0

B. Dose Selection

Since the amount of the sample available for the assay was limited, no dose rangefinding study was performed on this sample. Routinely, between 50 to 75% of the available extract will be used in the initial mutagenicity assay, with the remaining extract reserved for possible retesting and confirmation studies. The total amount of extractives obtained after processing 29.2 liters of the sample through XAD-2 resin was 13.5 mg in a volume of 0.5 ml DMSO. The deionized water (48 liters) passed through XAD-2 resin had a total extractives of 0.0 mg in a volume of 0.5 ml DMSO. The amount of the extractives obtained from the deionized water control was deducted from the total extractives of the test sample (13.5 mg - 0.0 mg = 13.5 mg) to calculate the doses used in the mutagenicity assay. Based on the

amount of available extractives, the test sample extract was assayed at six doses, 500, 251, 124, 50.0, 25.1 and 12.4 μg of extractives per plate using two plates per dose level.

C. Mutagenicity Assay

The dose levels selected for the mutagenicity assay ranged from 500 to 12.4 μg per plate in both the presence and absence of S9.

The mutagenicity assay results for **Raw Groundwater** are presented in Tables 1 through 4. These data were generated in two Experiments, 14544-B1 and 14544-B2. The data are presented as mean revertants per plate ± standard deviation for each treatment and control group (Tables 2 and 4) and as individual plate counts (Tables 1 and 3).

In Experiment 14544-B1 (Tables 1 and 2), all data were acceptable and positive increases in the number of histidine revertants per plate were observed with tester strain TA98 (3.6-fold) and TA100 (2.0-fold), only in the presence of S9. No positive increases were observed with either tester strain in the absence of S9. In order to confirm the observed increases, tester strains TA98 and TA100 were retested in the presence of S9 in Experiment 14544-B2.

In Experiment 14544-B2 (Tables 3 and 4), all data were acceptable and a positive increase in the number of histidine revertants per plate was observed with tester strain TA98 (4.5-fold) in the presence of S9. A positive increase was not observed with tester strain TA100 in the presence of S9 in the confirmatory assay.

The responses observed with tester strains TA98 and TA100 in the presence of S9 are presented graphically in Figures 1 and 2.

All criteria for a valid study were met.

CONCLUSIONS

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts indicate that under the conditions of this study, an extract of Johns Hopkins University's test article, Raw Groundwater, did cause a positive increase in the number of histidine revertants per plate with tester strain TA98 in the presence of S9 in two independent trials (3.6 and 4.5-fold). A positive increase was also observed with tester strain TA100 in the presence of S9 (2.0-fold) in the initial experiment. However, in the confirmatory assay, this positive increase could not be reproduced. No positive increases were observed with either tester strain in the absence of S9.

SECTION V. DATA TABLES

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TABLE 1
MUTAGENICITY ASSAY RESULTS
INDIVIDUAL PLATE COUNTS

EXPERIMENT ID: 14544-B1 — TEST ARTICLE ID: Raw Groundwater

DATE PLATED: 15-May-91 — VEHICLE: DMSO

DATE COUNTED: 20-May-91 — PLATING ALIQUOT: 50 µl

DOSE/PLATE		REVERTANTS PER PLATE TA98 1	TA98 2	TA98 3	TA100 1	TA100 2	TA100 3	BACKGROUND LAWN*
MICROSOMES: Rat Liver								
RESIN CONTROL		27	37		176	135		1
NEGATIVE CONTROL		36	41		115	103		1
VEHICLE CONTROL		29	42	38	117	108	104	1
TEST ARTICLE	12.4 µg	45	35		134	125		1
	25.1 µg	30	38		119	97		1
	50.0 µg	38	29		130	141		1
	124 µg	53	59		116	150		1
	251 µg	92	81		166	194		1
	500 µg	144	118		207	243		1
POSITIVE CONTROL **		1102	998		1313	1138		1
MICROSOMES: None								
RESIN CONTROL		15	10		98	95		1
NEGATIVE CONTROL		20	29		90	97		1
VEHICLE CONTROL		19	32	25	80	80	86	1
TEST ARTICLE	12.4 µg	19	28		80	82		1
	25.1 µg	15	23		110	89		1
	50.0 µg	24	24		88	80		1
	124 µg	22	20		104	94		1
	251 µg	41	26		94	89		1
	500 µg	24	24		95	88		1
POSITIVE CONTROL ***		136	119		443	496		1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal — 2 = slightly reduced — 3 = moderately reduced
4 = extremely reduced — 5 = absent — 6 = obscured by precipitate
sp = slight precipitate — mp = moderate precipitate (requires hand count) — hp = heavy precipitate (requires hand count)

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TABLE 2
MUTAGENICITY ASSAY RESULTS
SUMMARY OF TEST RESULTS

EXPERIMENT ID: 14544-B1 TEST ARTICLE ID: Raw Groundwater

DATE PLATED: 15-May-91 VEHICLE: DMSO

DATE COUNTED: 20-May-91 PLATING ALIQUOT: 50 µl

DOSE/PLATE	MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION TA98 MEAN	TA98 S.D.	TA100 MEAN	TA100 S.D.	BACKGROUND LAWN*
MICROSOMES: Rat Liver					
RESIN CONTROL	32	7	156	29	1
NEGATIVE CONTROL	39	4	109	8	1
VEHICLE CONTROL	36	7	[illegible]	7	1
TEST ARTICLE 12.4 µg	40	7	130	6	1
25.1 µg	44	6	108	16	1
50.0 µg	34	6	136	8	1
124 µg	56	4	133	24	1
251 µg	87	8	180	20	1
500 µg	131	18	225	25	1
POSITIVE CONTROL **	1050	74	1226	124	1
MICROSOMES: None					
RESIN CONTROL	13	4	97	2	1
NEGATIVE CONTROL	25	6	94	5	1
VEHICLE CONTROL	25	7	82	3	1
TEST ARTICLE 12.4 µg	24	6	81	1	1
25.1 µg	19	6	100	15	1
50.0 µg	24	0	84	6	1
124 µg	21	1	99	7	1
251 µg	34	11	92	4	1
500 µg	24	0	92	5	1
POSITIVE CONTROL ***	128	12	470	37	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

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TABLE 3
MUTAGENICITY ASSAY RESULTS
INDIVIDUAL PLATE COUNTS

EXPERIMENT ID: 14544-B2 | TEST ARTICLE ID: Raw Groundwater

DATE PLATED: 29-May-91 | VEHICLE: DMSO

DATE COUNTED: 03-Jun-91 | PLATING ALIQUOT: 50 µl

DOSE/PLATE		REVERTANTS PER PLATE TA98 1	TA98 2	TA98 3	TA100 1	TA100 2	TA100 3	BACKGROUND LAWN*
MICROSOMES: Rat Liver								
NEGATIVE CONTROL		25	26		128	123		1
VEHICLE CONTROL		17	11	32	126	125	95	1
TEST ARTICLE	12.5 µg	20	19		132	126		1
	25.0 µg	18	20		133	136		1
	50 0 µg	13	27		113	130		1
	125 µg	39	33		137	157		1
	250 µg	42	55		137	149		1
	500 µg	84	93		172	147		1
POSITIVE CONTROL **		1098	1224		1208	1251		1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

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TABLE 4
MUTAGENICITY ASSAY RESULTS
SUMMARY OF TEST RESULTS

EXPERIMENT ID: 14544-B2

TEST ARTICLE ID: Raw Groundwater

DATE PLATED: 29-May-91

VEHICLE: DMSO

DATE COUNTED: 03-Jun-91

PLATING ALIQUOT: 50 µl

DOSE/PLATE		MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION TA98 MEAN	TA98 S.D.	TA100 MEAN	TA100 S.D.	BACKGROUND LAWN*
MICROSOMES: Rat Liver						
NEGATIVE CONTROL		26	1	126	4	1
VEHICLE CONTROL		20	11	115	18	1
TEST ARTICLE	12.5 µg	20	1	129	4	1
	25.0 µg	19	1	135	2	1
	50.0 µg	20	10	122	12	1
	125 µg	36	4	147	14	1
	250 µg	49	9	143	8	1
	500 µg	89	6	160	18	1
POSITIVE CONTROL **		1161	89	1230	30	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

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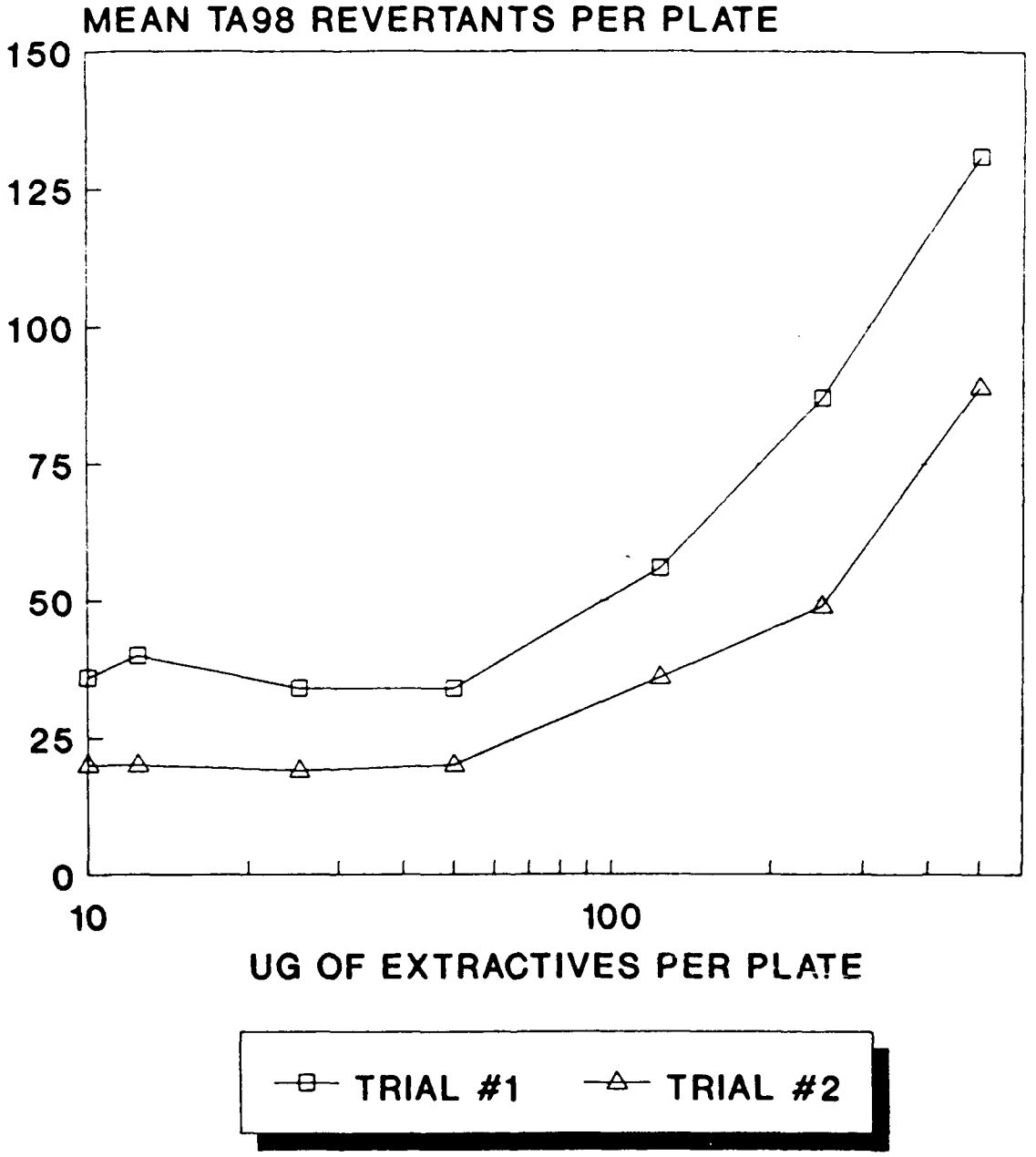
FIGURE 1
AMES TEST RESULTS
TESTER STRAIN TA98 WITH S9



14544-1-401X

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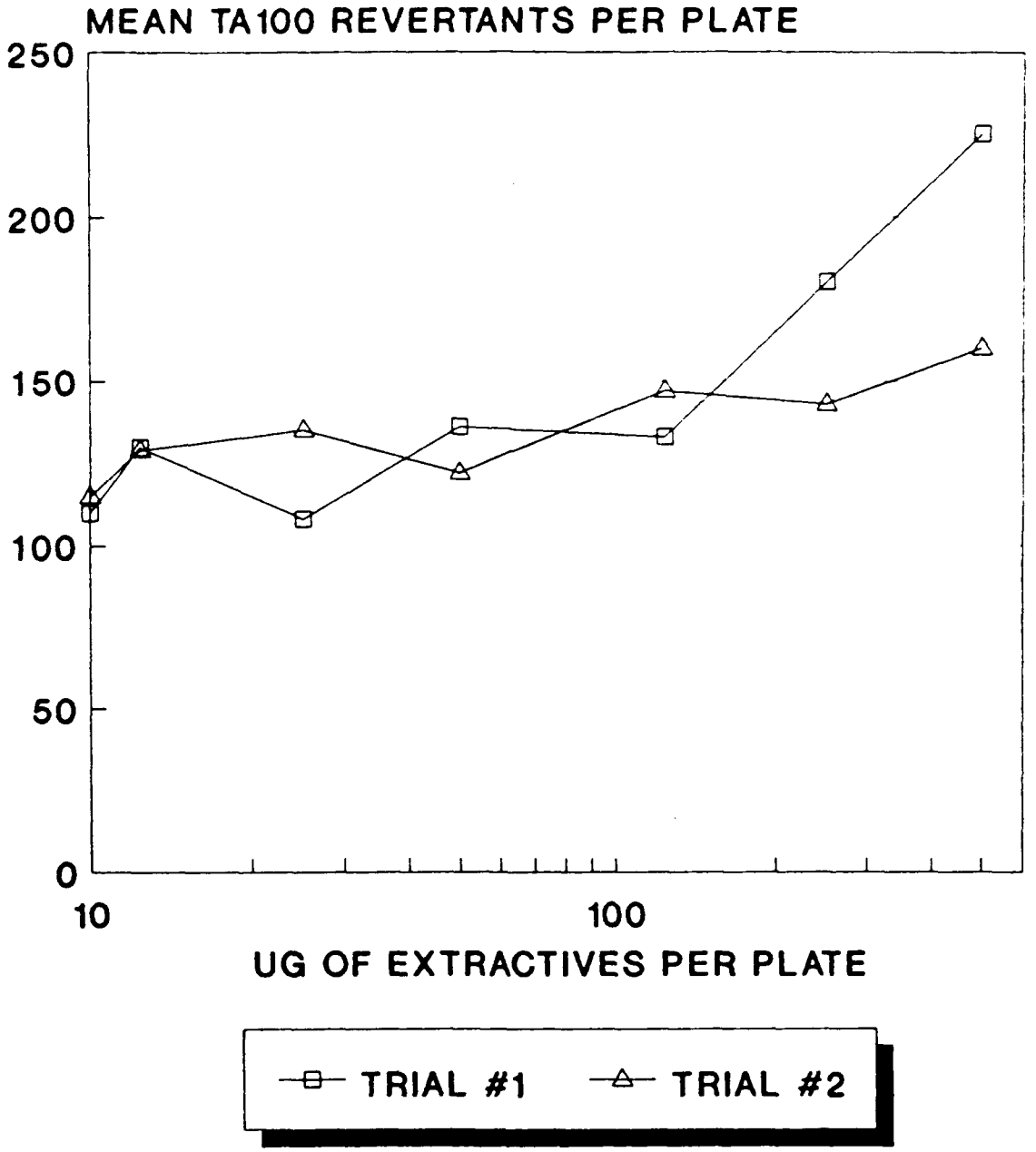
FIGURE 2
AMES TEST RESULTS
TESTER STRAIN TA100 WITH S9



14544-1-401X

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5516 NICHOLSON LANE
KENSINGTON MARYLAND 20895

a CORNING Laboratory Services Company

MUTAGENICITY TEST ON

UV-OXIDATION TREATED WATER

IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY
(AMES TEST)
MODIFIED FOR DIRECT WATER SAMPLES

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Hazleton Washington, Inc.
5516 Nicholson Lane
Kensington, Maryland 20895

LABORATORY PROJECT ID

HWA Study No.: 14545-0-401W

SUBMITTED TO

Johns Hopkins University
Applied Physics Laboratory
4800 Atwell Road
Shady Side, MD 20764

STUDY COMPLETION DATE

June 10, 1991

United States • Germany • Japan • France • United Kingdom
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QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples

STUDY NO.: 14545-0-401W

PROTOCOL NO.: 401W EDITION NO.: 16

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection - Date	Findings Reported	Auditor
Preparation of S9 Mix - 05/02/91	05/02/91	D. Wallace
Final Report Review - 06/07/91	06/07/91	D. Wallace

D. Wallace 6/7/91

Quality Assurance Unit Date Released

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COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160). To the best of the signer's knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The stability of the test article under the conditions of administration was the responsibility of the Sponsor. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of the HWA Division of Molecular and Cellular Services, 5516 Nicholson Lane, Kensington, Maryland 20895. After two years, these records will be transferred to permanent archives at Hazleton Washington, Vienna, Virginia.

SUBMITTED BY:

Study Director:

Timothy E. Lawlor, M.A.
Microbial Mutagenesis
Department of Molecular and Cellular Services

6.10.91
Study Completion Date

HAZLETON
WASHINGTON

TABLE OF CONTENTS

SECTION I. SUMMARY

INTRODUCTION AND CONCLUSIONS

SUMMARY

A. Introduction

At the request of Johns Hopkins University, Hazleton Washington, Inc. examined water sample UV-Oxidation Treated Water for mutagenic activity in the Salmonella/ Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor-induced rat liver.

The tester strains used in this study were TA98 and TA100. The assay was conducted using two plates per dose level in the presence and absence of microsomal enzyme. Six dose levels of the test article, 3.00, 2.00, 1.00, 0.500, 0.200 and .100 ml per plate, were tested in both the presence and absence of S[illegible]ong with the appropriate vehicle controls (three plates per dose), negative controls and positive controls.

B. Conclusions

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples indicate that under the conditions of this study, Johns Hopkins University's test article, UV-Oxidation Treated Water, did not cause a positive increase in the numbers of histidine revertants per plate with tester strains TA98 and TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

SECTION II. STUDY INFORMATION

STUDY INFORMATION

A. Sponsor: Johns Hopkins University

B. Test Article: UV-Oxidation Treated Water

1. Physical Description: clear colorless liquid

2. Date Received: 04/29/91

C. Type of Assay: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Modified for Direct Water Samples)

1. Protocol Number: HWA Protocol 401W, Edition 16

2. HWA Study Number: 14545-0-401W

D. Study Dates

1. Study Initiation Date: 04/29/91

2. Test Initiation in the Laboratory: 05/02/91

3. Test Completion in the Laboratory: 05/10/91

E. Study Personnel

Study Director: Timothy E. Lawlor, M.A.

Laboratory Supervisor: Michael S. Mecchi, B.S.

Technician: Theodora Brown

Technician: Sow Hoong Hon, B.S.

SECTION III. MATERIALS AND METHODS

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames et al (1975).

A. Media and Reagents

1. Top Agar for Selection of Histidine Revertants: Since different volumes of neat water sample will be added directly to the tubes containing top agar, it is necessary to prepare tubes containing different concentrations of top agar components (agar, NaCl, histidine and biotin), such that once the appropriate volume of water sample has been added, the top agar component concentration in all top agar tubes will be comparable. A series of top agar tubes was prepared as follows:

Top Agar Tube	Top Agar Component Concentration (%)	ml of 0.5mM Histidine/ Biotin Solution Added per ml of Top Agar	ml of Supplemented Top Agar Added per Tube	ml of Test Article Added per Tube
1.0 X	Agar 0.60			
	NaCl 0.50	0.07	3.0	0.1
1.0 X	Agar 0.60			
	NaCl 0.50	0.07	3.0	0.2
1.5 X	Agar 0.72			
	NaCl 0.60	0.08	2.5	0.5
2.0 X	Agar 0.90			
	NaCl 0.75	0.10	2.0	1.0
3.0 X	Agar 1.80			
	Nacl 1.50	0.20	1.0	2.0
4.0 X	Agar 2.40			
	NaCl 2.00	0.25	1.0	3.0

2. Minimal Bottom Agar: Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (W/V) agar and 0.2% (W/V) glucose.

3. Nutrient Broth: Nutrient Broth used for growing overnight cultures of the tester strains was Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

4. Exogenous Metabolic Activation

a. Liver Microsomal Enzymes - S9 Homogenate: S9 Liver homogenate for use in the mutagenicity assay, prepared as described below, was purchased from Molecular Toxicology, Inc., Annapolis, MD 21401, Batch 0327, 37.8 mg of protein per ml.

1) Species, Strain, Sex, Inducer: Liver microsomal enzymes were prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 (200 mg per ml in corn oil) at 500 mg/kg. Five days after i.p. injection with the Aroclor, the rats were sacrificed by decapitation, and their livers were excised.

2) Homogenate Preparation: The preparation of the microsomal enzyme fraction was carried out with sterile glassware and solutions at 6 ± 4°C. The livers were excised, weighed, and placed in a beaker containing 3 ml of 0.15M KCl per gram of wet liver, and homogenized. The homogenate was centrifuged at 9000 x g for 10 minutes. Small aliquots of the supernatant (referred to by Ames as the S9 fraction) were distributed into freezing ampules which were stored at ≤ -65°C.

3) S9 Characterization: The S9 homogenate was characterized (using the Ames Assay) for its ability to metabolize selected promutagens to their mutagenic forms, as described by deSerres and Shelby (1979).

b. S9 Mix: The S9 mix was prepared immediately before its use in the mutagenicity assay. One ml of the microsomal enzyme reaction mixture (S9 mix) contained the following components:

H_2O	0.70 ml
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 ml
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 ml
0.2M $MgCl_2$/0.825M KCl	0.04 ml
S9 Homogenate	0.10 ml
	1.00 ml

When required, 0.5 ml of the S9 mix was added to the soft agar overlay per plate.

B. Test System

1. Tester Strains

The tester strains used were the Salmonella typhimurium histidine auxotrophs TA98 and TA100 as described by Ames et al (1975).

TESTER STRAIN GENOTYPES

Histidine Mutation		Additional Mutations		
hisG46	hisD3052	LPS	Repair	R Factor
TA100	TA98	rfa	uvrB	+R

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the uvrB gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strain TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototropy) by frameshift mutagens. Tester strain TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

a. Source of Tester Strains: The tester strains in use at HWA were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

b. Storage of the Tester Strains

1) Frozen Permanent Stocks: Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (approximately 0.5 - 1.5 ml) at $\leq$-65°C.

2) Master Plates: Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 μM), biotin (3 μM), and ampicillin (25 μg/ml). Tester strain master plates were stored at 6 $\pm$ 4°C.

c. Preparation of Overnight Cultures: Overnight cultures were prepared by transferring a colony from the appropriate master plate to a flask containing culture medium. In order to assure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 $\pm$ 25 rpm; incubation, 37 $\pm$ 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began. Cultures were harvested once a predetermined turbidity was

reached as determined by a percent transmittance (%T) reading on a spectrophotometer. Overgrowth of cultures can result in their loss of sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached.

d. Confirmation of Tester Strain Genotypes: Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

1) rfa Wall Mutation: The presence of the rfa wall mutation was confirmed by demonstration of sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

2) pKM101 Plasmid R-factor: The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

3) Characteristic Number of Spontaneous Revertants: The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with 3.00 ml of deionized water on selective media.

C. Experimental Design

1. Mutagenicity Assay

The mutagenicity assay was performed using tester strains TA98 and TA100, both in the presence and absence of microsomal enzymes (S9 mix). Six dose levels of the test article, 3.00, 2.00, 1.00, 0.50, 0.20 and 0.10 ml per plate, were tested along with the appropriate vehicle, negative, and positive controls as specified by the New Jersey Department of Environmental Protection. If the test article exhibited toxicity in the assay in two or more doses, the assay would be repeated at lower concentrations.

a. Frequency and Route of Administration: The test system was exposed to the test article via the plate incorporation methodology originally described by Ames et al (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. All dose levels of test article, negative controls, and positive controls were plated in duplicate and the vehicle controls were plated in triplicate.

D. Controls

1. Positive Controls

Combinations of positive controls and tester strains plated concurrently with the assay are listed below.

POSITIVE CONTROL AND TESTER STRAIN COMBINATIONS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 µg
TA98	-	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	-	sodium azide	2.0 µg

a. Source and Grade of Positive Control Articles: 2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade; 2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98%; sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade.

2. Vehicle Controls

Sterilized deionized water (HWA Batch #193) vehicle controls were plated for both tester strains in the presence and absence of S9. The vehicle controls consisted of a 3.0 ml aliquot of deionized water (equal to the maximum aliquot of test article plated), along with an aliquot of the appropriate tester strain and an aliquot of S9 mix (when appropriate), plated on selective agar.

3. Negative Controls

Negative controls were plated for both tester strains in the presence and absence of S9. The negative controls consisted of an aliquot of the appropriate tester strain and S9 mix (when appropriate), plated on selective agar.

4. Sterility Controls

a. S9 Mix Sterility Determination: In order to determine the sterility of the S9 mix, a 0.5 ml aliquot was plated on selective agar.

E. Plating Procedures

The plating procedures employed are similar to those described by Ames et al (1975).

1. Test System Identification

Each plate was labeled with a code system which identified the test article, tester strain, test phase, dose level, and activation condition.

2. Test Article Plating Procedure

The S9 mix was prepared immediately before its use in the experimental procedure. An aliquot of tester strain (100 µl), the appropriate volume of vehicle or neat test article, and 0.5 ml of S9 mix (when necessary) were added to an appropriate amount and concentration of molten selective top agar (See III.A.1.) held in a 13 x 100 mm test tube at 45 ± 2°C. After vortexing, the mixture was overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 hours at 37 ± 2°C.

F. Scoring Plates

Plates which were not scored immediately after the 48 ± 8 hour incubation period were held at 6 ± 4°C until such time that scoring could occur.

1. Colony Counting

Revertant colonies for the negative, vehicle, and test article treated plates were counted by hand. The positive control plates were counted by automated colony counter.

2. Evaluation of the Bacterial Background Lawn

The condition of the background bacterial lawn was evaluated for evidence of test article cytotoxicity and precipitate. The cytotoxicity was scored relative to the vehicle control plate and is noted along with the revertant counts for all plates at that dose level on the data tables using the code system presented at the end of the Materials and Methods Section.

3. Analysis of the Data

For all replicate platings, the mean number of revertants per plate was calculated and the standard deviation around the mean was also calculated. The results of these calculations are presented in tabular form in the Data Tables Section of this report.

G. Criteria for Determination of a Valid Test

The following criteria must be met for the assay to be considered valid:

1. Tester Strain Integrity

a. rfa Wall Mutation: In order to demonstrate the presence of the deep rough mutation, tester strain cultures must exhibit sensitivity to crystal violet.

b. pKM101 Plasmid R-Factor: In order to demonstrate the presence of the pKM101 Plasmid R-factor, tester strains TA98 and TA100 must exhibit resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants: Tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The acceptable ranges are as follows:

TA98	8 - 60
TA100	60 - 240

d. Tester Strain Titers: In order to ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to 5.0×10^8 and/or have reached a target level of turbidity demonstrated to produce cultures with titers greater than or equal to 5.0×10^8.

e. Positive Control Values: All positive controls must exhibit at least a three-fold increase in the number of revertants per plate over the mean value for the vehicle control for the respective strain.

2. Cytotoxicity

a. Acceptable Number of Non-toxic Dose Levels: A minimum of three non-toxic dose levels are required to evaluate assay data.

H. Evaluation of Test Results

1. Tester Strains TA98 and TA100

For a test article to be considered positive, it must cause at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

I. References

Ames, B.N., J. McCann and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Research 31:347-364 (1975).

deSerres, F.J., and M.D. Shelby. Recommendations on Data Production and Analysis using the Salmonella/Microsome Mutagenicity Assay: Mutation Research 64:159-165 (1979).

Maron, D.M., and B. Ames. Revised Methods for the Salmonella Mutagenicity Test. Mutation Research 113:173-215 (1983).

Vogel, H.J., and D.M. Bonner. Acetylornithinase of E. coli: Partial Purification and Some Properties, J. Biol. Chem. 218:97-106 (1956).

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS OF BACKGROUND LAWN
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

SECTION IV. RESULTS AND CONCLUSIONS

RESULTS

A. Test Article Handling

The water sample, UV-Oxidation Treated Water, was stored refrigerated at 5 ± 3°C until used in the assay. The test article was filter sterilized using a 0.45 μm filter to remove any particulate matter and possible bacterial or other contaminant that would interfere with the assay.

B. Mutagenicity Assay

The dose levels selected for the mutagenicity assay were 3.00, 2.00, 1.00, 0.500, 0.200, and 0.100 ml of unconcentrated test article per plate in the presence and absence of S9, as specified by the New Jersey Department of Environmental Protection. The mutagenicity assay results for UV-Oxidation Treated Water are presented in Tables 1 and 2. These data were generated in Experiment 14545-B2 (in an initial experiment, 14545-B1, no data was generated due to the malfunction of an incubator). The data are presented as mean revertants per plate ± standard deviation for each treatment and control group (Table 2) and as individual plate counts (Table 1).

In Experiment 14545-B2, all data were acceptable and no positive increases in the number of histidine revertants per plate were observed.

All criteria for a valid study were met.

CONCLUSIONS

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples indicate that under the conditions of this study, Johns Hopkins University's test article, UV-Oxidation Treated Water, did not cause a positive increase in the number of histidine revertants per plate with tester strains TA98 and TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

SECTION V. DATA TABLES

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TABLE 1

INDIVIDUAL PLATE COUNTS

TEST ARTICLE ID: UV-Oxidation Treated Water

EXPERIMENT ID: 14545-B2 VEHICLE: deionized water

DATE PLATED: 02-May-91 DATE COUNTED: 09-May-91

		REVERTANTS PER PLATE						BACKGROUND LAWN*
DOSE/PLATE		TA98			TA100			
		1	2	3	1	2	3	
MICROSOMES: Rat Liver								
VEHICLE CONTROL		23	12	18	147	116	95	1
NEGATIVE CONTROL		34	31		157	144		1
TEST ARTICLE	0.100 ml	43	35		114	112		1
	0.200 ml	41	38		118	95		1
	0.500 ml	34	28		110	96		1
	1.00 ml	35	26		87	86		1
	2.00 ml	27	24		107	87		1
	3.00 ml	30	19		114	106		1
POSITIVE CONTROL **		1330	1231		1166	1037		1
MICROSOMES: None								
VEHICLE CONTROL		19	15	12	79	75	68	1
NEGATIVE CONTROL		17	14		68	62		1
TEST ARTICLE	0.100 ml	22	18		74	69		1
	0.200 ml	23	22		86	79		1
	0.500 ml	15	13		106	103		1
	1.00 ml	26	25		97	84		1
	2.00 ml	17	15		92	89		1
	3.00 ml	14	10		89	79		1
POSITIVE CONTROL ***		161	142		554	525		1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

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TABLE 2

SUMMARY OF TEST RESULTS

TEST ARTICLE ID: UV-Oxidation Treated Water

EXPERIMENT ID: 14545-B2 VEHICLE: deionized water

DATE PLATED: 02-May-91 DATE COUNTED: 09-May-91

	DOSE/PLATE	MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION TA98 MEAN	TA98 S.D.	TA100 MEAN	TA100 S.D.	BACKGROUND LAWN*
MICROSOMES: Rat Liver						
VEHICLE CONTROL		18	6	119	26	1
NEGATIVE CONTROL		33	2	151	9	1
TEST ARTICLE	0.100 ml	39	6	113	1	1
	0.200 ml	40	2	107	16	1
	0.500 ml	31	4	103	10	1
	1.00 ml	31	6	87	1	1
	2.00 ml	26	2	97	14	1
	3.00 ml	25	8	110	6	1
POSITIVE CONTROL **		1281	70	1102	91	1
MICROSOMES: None						
VEHICLE CONTROL		15	4	74	6	1
NEGATIVE CONTROL		16	2	65	4	1
TEST ARTICLE	0.100 ml	20	3	72	4	1
	0.200 ml	23	1	83	5	1
	0.500 ml	14	1	105	2	1
	1.00 ml	26	1	91	9	1
	2.00 ml	16	1	91	2	1
	3.00 ml	12	3	84	7	1
POSITIVE CONTROL ***		152	13	540	21	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

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a CORNING Laboratory Services Company

MUTAGENICITY TEST ON

AN EXTRACT OF THE WATER SAMPLE

UV-OXIDATION TREATED

IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY (AMES TEST) MODIFIED FOR XAD-2 RESIN EXTRACTS

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Hazleton Washington, Inc.
5516 Nicholson Lane
Kensington, Maryland 20895

LABORATORY PROJECT ID

HWA Study No.: 14545-1-401X

SUBMITTED TO

Johns Hopkins University
Applied Physics Laboratory
4800 Atwell Road
Shady Side, MD 20764

STUDY COMPLETION DATE

June 12, 1991

United States • Germany • Japan • France • United Kingdom
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QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts

STUDY NO.: 14545-1-401X

PROTOCOL NO.: 401X EDITION NO.: 17

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection - Date	Findings Reported	Auditor
Counting of Colonies - 05/20/91	05/20/91	M. Murphy
Final Report Review - 06/11/91	06/11/91	P. Postal

Path Postal 6·12·91

Quality Assurance Unit Date Released

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COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160). To the best of the signer's knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The stability of the test article under the conditions of administration was the responsibility of the Sponsor. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of the HWA Division of Molecular and Cellular Services, 5516 Nicholson Lane, Kensington, Maryland 20895. After two years, these records will be transferred to permanent archives at Hazleton Washington, Vienna, Virginia.

SUBMITTED BY:

Study Director:

Timothy E. Lawlor, M.A.
Microbial Mutagenesis
Department of Molecular and Cellular Toxicology

6·12·91
Study Completion Date

HAZLETON
WASHINGTON

TABLE OF CONTENTS

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SECTION I. SUMMARY

INTRODUCTION AND CONCLUSIONS

SUMMARY

A. Introduction

At the request of Johns Hopkins University, Hazleton Washington, Inc. examined an extract of the water sample, UV-Oxidation Treated, for mutagenic activity in the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver.

The tester strains used in this study were TA98 and TA100. The assay was conducted using two plates per dose level in the presence and absence of microsomal enzymes. Six dose levels of the test article were tested, from 75.0 to 1.26 μg per plate in both the presence and absence of S9. Vehicle controls (three plates per dose), negative controls, resin controls and positive controls were plated as part of the assay.

The doses tested in the mutagenicity assay were selected based on the amount of extractable organics recovered from the test article.

B. Conclusions

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts indicate that under the conditions of this study, an extract of Johns Hopkins University's test article, UV-Oxidation Treated, did not cause a positive increase in the number of histidine revertants per plate with tester strain TA98 or TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver.

SECTION II. STUDY INFORMATION

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STUDY INFORMATION

A. Sponsor: Johns Hopkins University

B. Test Article: UV-Oxidation Treated

1. Physical Description, Test Article: clear colorless liquid

2. Date Received: 04/29/91

3. Physical Description, Extract: pale amber liquid

C. Type of Assay: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts

1. Protocol Number: HWA Protocol 401X, Edition 17

2. HWA Study Number: 14545-1-401X

D. Study Dates

1. Study Initiation Date: 04/29/91

2. Experimental Start: 05/15/91

3. Experimental Termination: 05/20/91

E. Study Personnel

Study Director: Timothy E. Lawlor, M.A.

Laboratory Supervisor: Michael S. Mecchi, B.S.

Research Assistant: Ralph S. McCrea, B.S.

Technician: Sow Hoong Hon, B.S.

SECTION III. MATERIALS AND METHODS

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames et al (1975).

MATERIALS

A. Tester Strains

The tester strains used were the Salmonella typhimurium histidine auxotrophs TA98 and TA100 as described by Ames et al (1975). The specific genotypes of these strains are shown in Table 1.

TABLE 1. TESTER STRAIN GENOTYPES

Histidine Mutation		Additional Mutations		
hisG46	hisD3052	LPS	Repair	R Factor
TA100	TA98	rfa	uvrB	+R

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the uvrB gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strain TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototropy) by frameshift mutagens. Tester strain TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

1. Source of Tester Strains

The tester strains in use at HWA were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

2. Storage of the Tester Strains

a. Frozen Permanent Stocks

Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (approximately 0.5 - 1.5 ml) at ≤-70°C.

b. Master Plates

Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 μM), biotin (3 μM), and for strains containing the R-factor, ampicillin (25 μg/ml). Tester strain master plates were stored at 5 ± 3°C.

3. Preparation of Overnight Cultures

a. Inoculation

Overnight cultures for use in all testing procedures were inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 ± 25 rpm; incubation, 37 ± 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began.

b. Harvest

To ensure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring of culture turbidity. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance (%T) reading on a spectrophotometer. This target turbidity ensures that cultures have reached a density of at least 0.5 X 10^9 cells per ml and that the cultures have not overgrown. Overgrown (stationary) cultures may exhibit decreased sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached and were placed at 5 ± 3°C.

4. Confirmation of Tester Strain Genotypes

Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

a. rfa Wall Mutation

The presence of the rfa wall mutation was confirmed by demonstration of the cultures sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

b. pKM101 Plasmid R-factor

The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

c. Characteristic Number of Spontaneous Revertants

The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with the appropriate vehicle on selective media.

5. Tester Strain Media

a. Culturing Broth

The broth used to grow overnight cultures of the tester strains was Vogel-Bonner salt solution supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

b. Agar Plates

Bottom agar (25 ml per 15 x 100 mm petri dish) was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (W/V) agar and 0.2% (W/V) glucose.

c. Overlay Agar for Selection of Histidine Revertants

Overlay (top) agar was prepared with 0.7% agar (W/V) and 0.5% NaCl (W/V) and was supplemented with 10 ml of 0.5 mM histidine/biotin solution per 100 ml agar. When S9 mix is required, 2.0 ml of the supplemented top agar is used in the overlay. However, when S9 is not required, water is added to the supplemented top agar (0.5 ml of water per 2 ml of supplemented top agar) and the resulting 2.5 ml of diluted supplemented top agar is used for the overlay. This dilution ensures that the final top agar and amino acid

supplement concentrations remain the same both in the presence and absence of S9.

B. Liver Microsomal Enzyme Reaction Mixture (S9 Mix)

1. S9 Homogenate

Liver microsomal enzymes (S9 homogenate) were purchased from Molecular Toxicology, Inc., Annapolis, MD 20401, Batch 0327 (37.8 mg of protein per ml). The homogenate was prepared from male Sprague-Dawley rats that had been injected (i.p.) with Aroclor™ 1254 (200 mg per ml in corn oil) at 500 mg/kg as described by Ames et al, 1975.

2. S9 Mix

The S9 mix was prepared immediately prior to its use in any experimental procedure. The S9 mix contained the components indicated in Table 2.

TABLE 2. S9 MIX COMPONENTS

H_2O	0.70 ml
1M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 ml
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 ml
0.825M KCl/0.2M $MgCl_2$	0.04 ml
S9 Homogenate	0.10 ml
	1.00 ml

C. Controls

1. Vehicle Controls

Dimethylsulfoxide (DMSO, CAS# 67-68-5, Sigma Chemical Co., Lot 30H0608, 99+%) vehicle controls were plated for both tester strains in the presence and absence of S9. The vehicle control was plated, using a 50 µl aliquot of DMSO (equal to the maximum aliquot of test article dilution plated), along with a 100 µl aliquot of the appropriate tester strain and a 500 µl aliquot of S9 (when necessary), on selective agar.

2. Negative Controls

Negative controls were plated for both tester strains in the presence and absence of S9. The negative controls consisted of a 100 µl aliquot of the appropriate tester strain and S9 (when appropriate) plated on selective agar.

3. Resin Controls

Resin controls were plated for both tester strains in the presence and absence of S9. A sample of deionized water was extracted and concentrated using the same XAD-2 resin adsorption procedure used for the test article. The resin control was tested at a single concentration. The resin controls consisted of a 50 µl aliquot of the resin control extract, a 100 µl aliquot of tester strain and an aliquot of S9 (when appropriate) plated on selective agar.

4. Positive Controls

The combinations of positive controls, activation condition and tester strains plated concurrently with the assay are indicated in Table 3.

TABLE 3. POSITIVE CONTROLS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 µg
TA98	-	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	-	sodium azide	2.0 µg

a. Source and Grade of Positive Control Articles

2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade; 2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98%; sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade.

5. Sterility Controls

a. S9 Mix

The S9 mix was checked for sterility by plating 0.5 ml on selective agar.

METHODS

A. Mutagenicity Assay

1. Design

The assay was performed using tester strains TA98 and TA100 both in the presence and absence of S9 mix. Six dose levels of the test

article extract were tested along with the appropriate vehicle, negative, resin and positive controls. The dose levels tested were selected based on the amount of extractable organics recovered in the extraction procedure.

2. Frequency and Route of Administration

The tester strains were exposed to the test article via the plate incorporation methodology originally described by Ames et al (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. In the plate incorporation methodology, the test article, the tester strain and the S9 mix (where appropriate) are combined in molten agar which is overlaid onto a minimal agar plate. Following incubation at 37 ± 2°C for 48 ± 8 hr, revertant colonies will be counted. All dose levels of test article, negative controls, resin controls and positive controls were plated in duplicate, and the vehicle controls were plated in triplicate.

B. Plating Procedures

Each plate was labeled with a code which identified the test article, test phase, tester strain, activation condition and dose level. The S9 mix and dilutions of the test article were prepared immediately prior to their use.

When S9 mix was not required, 100 µl of tester strain and 50 µl of vehicle or test article dose was added to 2.5 ml of molten selective top agar (maintained at 45 ± 2°C). When S9 mix was required, 500 µl of S9 mix, 100 µl of tester strain and 50 µl of vehicle or test article dose was added to 2.0 ml of molten selective top agar. After the required components had been added, the mixture was vortexed and overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 hr at 37 ± 2°C. Positive controls and the resin control were plated using a 50 µl plating aliquot.

C. Scoring the Plates

Plates which were not evaluated immediately following the incubation period were held at 5 ± 3°C until such time that colony counting and bacterial background lawn evaluation could take place.

1. Bacterial Background Lawn Evaluation

The condition of the bacterial background lawn was evaluated for evidence of cytotoxicity and test article precipitate. Evidence of cytotoxicity was scored relative to the vehicle control plate and was recorded along with the revertant counts for all plates at that dose level on the data tables using the code system presented at the end of the Materials and Methods Section.

2. Counting Revertant Colonies

The number of revertant colonies per plate for the vehicle controls and all plates containing test article were counted manually. The number of revertant colonies per plate for the positive controls were counted by automated colony counter.

D. Analysis of Data

For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The results of these calculations are presented in tabular form in the Data Tables Section of this report.

EVALUATION OF TEST RESULTS

Before assay data can be evaluated, the criteria for a valid assay must be met.

A. Criteria For A Valid Assay

The following criteria are used to determine a valid assay:

1. Tester Strain Integrity

a. rfa Wall Mutation

To demonstrate the presence of the rfa wall mutation, tester strain cultures must exhibit sensitivity to crystal violet.

b. pKM101 Plasmid

To demonstrate the presence of the R-factor plasmid, pKM101, cultures of tester strains TA98 and TA100 must exhibit resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants

To demonstrate the requirement for histidine, the tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable ranges for the vehicle controls are as follows:

TA98	8 - 60
TA100	60 - 240

d. Tester Strain Culture Density

To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures must be greater than or equal to 0.5×10^9 bacteria per ml and/or have reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 0.5×10^9 bacteria per ml.

e. Positive Control Values

(1) Positive Control Values in the Absence of S9 Mix

To demonstrate that the tester strains are capable of identifying a mutagen, the mean value of a positive control for a respective tester strain must exhibit at least a 3-fold increase over the mean value of the vehicle control for that strain.

(2) Positive Control Values in the Presence of S9 Mix (S9 Mix Integrity)

To demonstrate that the S9 mix is capable of metabolizing a promutagen to its mutagenic form(s), the mean value of the positive control for a respective tester strain in the presence of the S9 mix must exhibit at least a 3-fold increase over the mean value of the vehicle control for that strain.

An acceptable positive control in the presence of S9 for a specific strain will be evaluated as having demonstrated both the integrity of the S9 mix and the ability of the tester strain to detect a mutagen.

2. Cytotoxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data.

B. Criteria For A Positive Response

Once the criteria for a valid assay have been met, responses observed in the assay are evaluated as follows:

1. Tester Strains TA98 and TA100

For a test article to be considered positive, it must produce at least a 2-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

RECORDS TO BE MAINTAINED

All raw data, reports, protocols and modifications will be maintained by the Department of Molecular and Cellular Toxicology of HWA for a period of up to two years following submission of the final report to the Sponsor. After two years, all raw data and reports will be transferred to the HWA archives for permanent storage.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Research 31:347-364 (1975).

Maron, D.M., and Ames, B. Revised Methods for the Salmonella Mutagenicity Test. Mutation Research 113:173-215 (1983).

Vogel, H.J., and D.M. Bonner. Acetylornithinase of E. coli: Partial Purification and Some Properties, J. Biol. Chem. 218:97-106 (1956).

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS OF BACKGROUND LAWN
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

SECTION IV. RESULTS AND CONCLUSIONS

RESULTS

A. Test Article Handling

The water sample, UV-Oxidation Treated, was held at 5 ± 3°C until extracted and concentrated by the XAD-2 resin adsorption as described in the "Environmental Water Sample Processing Protocol No. 12, October, 1989."

The test article (36 liters) was passed through XAD-2 resin and solvent exchanged to dimethylsulfoxide (DMSO) using the procedure described below. Deionized water (48 liters) was also processed in the same manner as an internal control for the extraction procedure (resin control).

A chromatographic column (22 mm I.D.) was slurry-packed with 15 g of XAD-2 resin and washed with acetone (CAS# 67-64-1, Fisher Scientific, Lot 900324) and water. All extraction procedures were performed at 5 ± 3°C. The test article was passed through the XAD-2 column using gravity flow at a rate of 60 to 80 drops/min. Material adsorbed to the column was eluted with acetone and methylene chloride (CAS# 75-09-2, Fisher Scientific, Lot 890625). The eluate was reduced in volume using a rotary evaporator. Duplicate aliquots of the concentrate were transferred to tared aluminum weighing dishes, the solvent allowed to evaporate off, and the amount of material extracted from the water samples determined gravimetrically. The remainder of the extract was solvent exchanged into 0.5 ml dimethylsulfoxide (DMSO, CAS# 67-68-5, Sigma Chemical Co., Lot 30H0608, 99+%).

Extractable Organics

	Sample Volume	Extractives mg/0.5 ml
UV-Oxidation Treated	36 Liters	1.8
Resin Control (deionized water)	48 Liters	0.0

B. Dose Selection

Since the amount of the sample available for the assay was limited, no dose rangefinding study was performed on this sample. Routinely, between 50 to 75% of the available extract will be used in the initial mutagenicity assay, with the remaining extract reserved for possible retesting and confirmation studies. The total amount of extractives obtained after processing 36 liters of the sample through XAD-2 resin was 1.8 mg in a volume of 0.5 ml DMSO. The deionized water (48 liters) passed through XAD-2 resin had a total extractives of 0.0 mg in a volume of 0.5 ml DMSO. The amount of the extractives obtained from the deionized water control was deducted from the total extractives of the test sample (1.8 mg - 0.0 mg = 1.8 mg) to

calculate the doses used in the mutagenicity assay. Based on the amount of available extractives, the test sample extract was assayed at six doses, 75.0, 24.9, 12.6, 7.50, 2.49 and 1.26 μg of extractives per plate using two plates per dose level.

C. Mutagenicity Assay

The dose levels selected for the mutagenicity assay ranged from 75.0 to 1.26 μg per plate in both the presence and absence of S9.

The mutagenicity assay results for UV-Oxidation Treated are presented in Tables 1 and 2. These data were generated in Experiment 14545-B1. The data are presented as mean revertants per plate ± standard deviation for each treatment and control group (Table 2) and as individual plate counts (Table 1).

In Experiment 14545-B1, all data were acceptable and no positive increases in the number of histidine revertants per plate were observed with tester strains TA98 or TA100 in either the presence or absence of S9.

All criteria for a valid study were met.

CONCLUSIONS

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts indicate that under the conditions of this study, an extract of Johns Hopkins University's test article, UV-Oxidation Treated, did not cause a positive increase in the number of histidine revertants per plate with tester strains TA98 and TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver.

SECTION V. DATA TABLES

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TABLE 1

INDIVIDUAL PLATE COUNTS

EXPERIMENT ID: 14545-B1 TEST ARTICLE ID: UV-Oxidation Treated

DATE PLATED: 15-May-91 VEHICLE: DMSO

DATE COUNTED: 20-May-91 PLATING ALIQUOT: 50 µl

DOSE/PLATE		REVERTANTS PER PLATE TA98 1	TA98 2	TA98 3	TA100 1	TA100 2	TA100 3	BACKGROUND LAWN*
MICROSOMES: Rat Liver								
RESIN CONTROL		27	37		176	135		1
NEGATIVE CONTROL		30	31		100	101		1
VEHICLE CONTROL		31	33	29	103	101	92	1
TEST ARTICLE	1.26 µg	39	41		104	90		1
	2.49 µg	38	39		106	123		1
	7.50 µg	33	31		102	106		1
	12.6 µg	38	50		114	150		1
	24.9 µg	34	44		133	121		1
	75.0 µg	48	35		136	129		1
POSITIVE CONTROL **		934	1133		1085	1200		1
MICROSOMES: None								
RESIN CONTROL		15	10		98	95		1
NEGATIVE CONTROL		28	23		75	61		1
VEHICLE CONTROL		23	20	27	67	83	86	1
TEST ARTICLE	1.26 µg	17	18		80	82		1
	2.49 µg	26	19		78	87		1
	7.50 µg	17	17		70	80		1
	12.6 µg	30	19		112	82		1
	24.9 µg	20	22		95	119		1
	75.0 µg	7	10		41	54		3
POSITIVE CONTROL ***		120	190		375	429		1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal 2 = slightly reduced 3 = moderately reduced
4 = extremely reduced 5 = absent 6 = obscured by precipitate
sp = slight precipitate mp = moderate precipitate (requires hand count) hp = heavy precipitate (requires hand count)

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TABLE 2

SUMMARY OF TEST RESULTS

EXPERIMENT ID: 14545-B1 TEST ARTICLE ID: UV-Oxidation Treated

DATE PLATED: 15-May-91 VEHICLE: DMSO

DATE COUNTED: 20-May-91 PLATING ALIQUOT: 50 µl

	DOSE/PLATE	MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATIONS TA98 MEAN	TA98 S.D.	TA100 MEAN	TA100 S.D.	BACKGROUND LAWN*
MICROSOMES: Rat Liver						
RESIN CONTROL		32	7	156	29	1
NEGATIVE CONTROL		31	1	101	1	1
VEHICLE CONTROL		31	2	99	6	1
TEST ARTICLE	1.26 µg	40	1	97	10	1
	2.49 µg	39	1	115	12	1
	7.50 µg	32	1	104	3	1
	12.6 µg	44	8	132	25	1
	24.9 µg	39	7	127	8	1
	75.0 µg	42	9	133	5	1
POSITIVE CONTROL **		1034	141	1143	81	1
MICROSOMES: None						
RESIN CONTROL		13	4	97	2	1
NEGATIVE CONTROL		26	4	68	10	1
VEHICLE CONTROL		23	4	79	10	1
TEST ARTICLE	1.26 µg	18	1	81	1	1
	2.49 µg	23	5	83	6	1
	7.50 µg	17	0	75	7	1
	12.6 µg	25	8	97	21	1
	24.9 µg	21	1	107	17	1
	75.0 µg	9	2	48	9	3
POSITIVE CONTROL ***		155	49	402	38	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal 2 = slightly reduced 3 = moderately reduced
4 = extremely reduced 5 = absent 6 = obscured by precipitate
sp = slight precipitate mp = moderate precipitate (requires hand count) hp = heavy precipitate (requires hand count)

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a CORNING Laboratory Services Company

MUTAGENICITY TEST ON

CARBON TREATED WATER

IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY (AMES TEST) MODIFIED FOR DIRECT WATER SAMPLES

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Hazleton Washington, Inc.
5516 Nicholson Lane
Kensington, Maryland 20895

LABORATORY PROJECT ID

HWA Study No.: 14546-0-401W

SUBMITTED TO

Johns Hopkins University
Applied Physics Laboratory
4800 Atwell Road
Shady Side, MD 20764

STUDY COMPLETION DATE

June 6, 1991

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HAZLETON
WASHINGTON

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples

STUDY NO.: 14546-0-401W

PROTOCOL NO.: 401W EDITION NO.: 16

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection - Date	Findings Reported	Auditor
Counting of Colonies - 05/09/91	05/09/91	M. Murphy
Final Report Review - 06/06/91	06/06/91	P. Postal

Patti Postal 6·6·91

Quality Assurance Unit Date Released

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COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160). To the best of the signer's knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The stability of the test article under the conditions of administration was the responsibility of the Sponsor. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of the HWA Division of Molecular and Cellular Services, 5516 Nicholson Lane, Kensington, Maryland 20895. After two years, these records will be transferred to permanent archives at Hazleton Washington, Vienna, Virginia.

SUBMITTED BY:

Study Director:

Timothy E. Lawlor, M.A.
Microbial Mutagenesis
Department of Molecular and Cellular Services

6·6·91
Study Completion Date

HAZLETON
WASHINGTON

TABLE OF CONTENTS

SECTION L SUMMARY

INTRODUCTION AND CONCLUSIONS

SUMMARY

A. Introduction

At the request of Johns Hopkins University, Hazleton Washington, Inc. examined water sample Carbon Treated Water for mutagenic activity in the Salmonella/ Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor-induced rat liver.

The tester strains used in this study were TA98 and TA100. The assay was conducted using two plates per dose level in the presence and absence of microsomal enzymes. Six dose levels of the test article, 3.00, 2.00, 1.00, 0.500, 0.200 and 0.100 ml per plate, were tested in both the presence and absence of S9 along with the appropriate vehicle controls (three plates per dose), negative controls and positive controls.

B. Conclusions

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples indicate that under the conditions of this study, Johns Hopkins University's test article, Carbon Treated Water, did not cause a positive increase in the numbers of histidine revertants per plate with tester strains TA98 and TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

SECTION II. STUDY INFORMATION

STUDY INFORMATION

A. Sponsor: Johns Hopkins University

B. Test Article: Carbon Treated Water

1. Physical Description: clear colorless liquid

2. Date Received: 05/01/91

C. Type of Assay: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples

1. Protocol Number: HWA Protocol 401W, Edition 16

2. HWA Study Number: 14546-0-401W

D. Study Dates

1. Study Initiation Date: 05/01/91

2. Test Initiation in the Laboratory: 05/02/91

3. Test Completion in the Laboratory: 05/09/91

E. Study Personnel

Study Director: Timothy E. Lawlor, M.A.

Laboratory Supervisor: Michael S. Mecchi, B.S.

Technician: Theodora Brown

Technician: Sow Hoong Hon, B.S.

SECTION III. MATERIALS AND METHODS

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames et al (1975).

A. Media and Reagents

1. Top Agar for Selection of Histidine Revertants: Since different volumes of neat water sample will be added directly to the tubes containing top agar, it is necessary to prepare tubes containing different concentrations of top agar components (agar, NaCl, histidine and biotin), such that once the appropriate volume of water sample has been added, the top agar component concentration in all top agar tubes will be comparable. A series of top agar tubes was prepared as follows:

Top Agar Tube	Top Agar Component Concentration (%)		ml of 0.5mM Histidine/ Biotin Solution Added per ml of Top Agar	ml of Supplemented Top Agar Added per Tube	ml of Test Article Added per Tube
1.0 X	Agar	0.60			
	NaCl	0.50	0.07	3.0	0.1
1.0 X	Agar	0.60			
	NaCl	0.50	0.07	3.0	0.2
1.5 X	Agar	0.72			
	NaCl	0.60	0.08	2.5	0.5
2.0 X	Agar	0.90			
	NaCl	0.75	0.10	2.0	1.0
3.0 X	Agar	1.80			
	Nacl	1.50	0.20	1.0	2.0
4.0 X	Agar	2.40			
	NaCl	2.00	0.25	1.0	3.0

2. Minimal Bottom Agar: Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (W/V) agar and 0.2% (W/V) glucose.

3. Nutrient Broth: Nutrient Broth used for growing overnight cultures of the tester strains was Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

4. Exogenous Metabolic Activation

a. Liver Microsomal Enzymes - S9 Homogenate: S9 Liver homogenate for use in the mutagenicity assay, prepared as described below, was purchased from Molecular Toxicology, Inc., Annapolis, MD 21401, Batch 0327, 37.8 mg of protein per ml.

1) Species, Strain, Sex, Inducer: Liver microsomal enzymes were prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 (200 mg per ml in corn oil) at 500 mg/kg. Five days after i.p. injection with the Aroclor, the rats were sacrificed by decapitation, and their livers were excised.

2) Homogenate Preparation: The preparation of the microsomal enzyme fraction was carried out with sterile glassware and solutions at 6 ± 4°C. The livers were excised, weighed, and placed in a beaker containing 3 ml of 0.15M KCl per gram of wet liver, and homogenized. The homogenate was centrifuged at 9000 x g for 10 minutes. Small aliquots of the supernatant (referred to by Ames as the S9 fraction) were distributed into freezing ampules which were stored at ≤ -65°C.

3) S9 Characterization: The S9 homogenate was characterized (using the Ames Assay) for its ability to metabolize selected promutagens to their mutagenic forms, as described by deSerres and Shelby (1979).

b. S9 Mix: The S9 mix was prepared immediately before its use in the mutagenicity assay. One ml of the microsomal enzyme reaction mixture (S9 mix) contained the following components:

H_2O	0.70 ml
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 ml
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 ml
0.2M $MgCl_2$/0.825M KCl	0.04 ml
S9 Homogenate	0.10 ml
	1.00 ml

When required, 0.5 ml of the S9 mix was added to the soft agar overlay per plate.

B. Test System

1. Tester Strains

The tester strains used were the Salmonella typhimurium histidine auxotrophs TA98 and TA100 as described by Ames et al (1975).

TESTER STRAIN GENOTYPES

Histidine Mutation		Additional Mutations		
hisG46	hisD3052	LPS	Repair	R Factor
TA100	TA98	rfa	uvrB	+R

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the uvrB gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strain TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototropy) by frameshift mutagens. Tester strain TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

a. Source of Tester Strains: The tester strains in use at HWA were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

b. Storage of the Tester Strains

1) Frozen Permanent Stocks: Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (approximately 0.5 - 1.5 ml) at $\leq$-65°C.

2) Master Plates: Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 μM), biotin (3 μM), and ampicillin (25 μg/ml). Tester strain master plates were stored at 6 $\pm$ 4°C.

c. Preparation of Overnight Cultures: Overnight cultures were prepared by transferring a colony from the appropriate master plate to a flask containing culture medium. In order to assure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 $\pm$ 25 rpm; incubation, 37 $\pm$ 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began. Cultures were harvested once a predetermined turbidity was

reached as determined by a percent transmittance (%T) reading on a spectrophotometer. Overgrowth of cultures can result in their loss of sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached.

d. Confirmation of Tester Strain Genotypes: Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

1) rfa Wall Mutation: The presence of the rfa wall mutation was confirmed by demonstration of sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

2) pKM101 Plasmid R-factor: The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

3) Characteristic Number of Spontaneous Revertants: The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with 3.00 ml of deionized water on selective media.

C. Experimental Design

1. Mutagenicity Assay

The mutagenicity assay was performed using tester strains TA98 and TA100, both in the presence and absence of microsomal enzymes (S9 mix). Six dose levels of the test article, 3.00, 2.00, 1.00, 0.50, 0.20 and 0.10 ml per plate, were tested along with the appropriate vehicle, negative, and positive controls as specified by the New Jersey Department of Environmental Protection. If the test article exhibited toxicity in the assay in two or more doses, the assay would be repeated at lower concentrations.

a. Frequency and Route of Administration: The test system was exposed to the test article via the plate incorporation methodology originally described by Ames et al (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. All dose levels of test article, negative controls, and positive controls were plated in duplicate and the vehicle controls were plated in triplicate.

D. Controls

1. Positive Controls

Combinations of positive controls and tester strains plated concurrently with the assay are listed below.

POSITIVE CONTROL AND TESTER STRAIN COMBINATIONS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 μg
TA98	-	2-nitrofluorene	1.0 μg
TA100	+	2-aminoanthracene	2.5 μg
TA100	-	sodium azide	2.0 μg

a. Source and Grade of Positive Control Articles: 2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade; 2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98%; sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade.

2. Vehicle Controls

Sterilized deionized water (HWA Batch #193) vehicle controls were plated for both tester strains in the presence and absence of S9. The vehicle controls consisted of a 3.0 ml aliquot of deionized water (equal to the maximum aliquot of test article plated), along with an aliquot of the appropriate tester strain and an aliquot of S9 mix (when appropriate), plated on selective agar.

3. Negative Controls

Negative controls were plated for both tester strains in the presence and absence of S9. The negative controls consisted of an aliquot of the appropriate tester strain and S9 mix (when appropriate), plated on selective agar.

4. Sterility Controls

a. S9 Mix Sterility Determination: In order to determine the sterility of the S9 mix, a 0.5 ml aliquot was plated on selective agar.

E. Plating Procedures

The plating procedures employed are similar to those described by Ames et al (1975).

1. Test System Identification

Each plate was labeled with a code system which identified the test article, tester strain, test phase, dose level, and activation condition.

2. Test Article Plating Procedure

The S9 mix was prepared immediately before its use in the experimental procedure. An aliquot of tester strain (100 µl), the appropriate volume of vehicle or neat test article, and 0.5 ml of S9 mix (when necessary) were added to an appropriate amount and concentration of molten selective top agar (See III.A.1.) held in a 13 x 100 mm test tube at 45 ± 2°C. After vortexing, the mixture was overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 hours at 37 ± 2°C.

F. Scoring Plates

Plates which were not scored immediately after the 48 ± 8 hour incubation period were held at 6 ± 4°C until such time that scoring could occur.

1. Colony Counting

Revertant colonies for the negative, vehicle, and test article treated plates were counted by hand. The positive control plates were counted by automated colony counter.

2. Evaluation of the Bacterial Background Lawn

The condition of the background bacterial lawn was evaluated for evidence of test article cytotoxicity and precipitate. The cytotoxicity was scored relative to the vehicle control plate and is noted along with the revertant counts for all plates at that dose level on the data tables using the code system presented at the end of the Materials and Methods Section.

3. Analysis of the Data

For all replicate platings, the mean number of revertants per plate was calculated and the standard deviation around the mean was also calculated. The results of these calculations are presented in tabular form in the Data Tables Section of this report.

G. Criteria for Determination of a Valid Test

The following criteria must be met for the assay to be considered valid:

1. Tester Strain Integrity

a. rfa Wall Mutation: In order to demonstrate the presence of the deep rough mutation, tester strain cultures must exhibit sensitivity to crystal violet.

b. pKM101 Plasmid R-Factor: In order to demonstrate the presence of the pKM101 Plasmid R-factor, tester strains TA98 and TA100 must exhibit resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants: Tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The acceptable ranges are as follows:

TA98	8 - 60
TA100	60 - 240

d. Tester Strain Titers: In order to ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to 5.0×10^8 and/or have reached a target level of turbidity demonstrated to produce cultures with titers greater than or equal to 5.0×10^8.

e. Positive Control Values: All positive controls must exhibit at least a three-fold increase in the number of revertants per plate over the mean value for the vehicle control for the respective strain.

2. Cytotoxicity

a. Acceptable Number of Non-toxic Dose Levels: A minimum of three non-toxic dose levels are required to evaluate assay data.

H. Evaluation of Test Results

1. Tester Strains TA98 and TA100

For a test article to be considered positive, it must cause at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

I. References

Ames, B.N., J. McCann and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Research 31:347-364 (1975).

deSerres, F.J., and M.D. Shelby. Recommendations on Data Production and Analysis using the Salmonella/Microsome Mutagenicity Assay: Mutation Research 64:159-165 (1979).

Maron, D.M., and B. Ames. Revised Methods for the Salmonella Mutagenicity Test. Mutation Research 113:173-215 (1983).

Vogel, H.J., and D.M. Bonner. Acetylornithinase of E. coli: Partial Purification and Some Properties, J. Biol. Chem. 218:97-106 (1956).

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS OF BACKGROUND LAWN
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

SECTION IV. RESULTS AND CONCLUSIONS

RESULTS

A. Test Article Handling

The water sample, Carbon Treated Water, was stored refrigerated at 5 ± 3°C until used in the assay. The test article was filter sterilized using a 0.45 µm filter to remove any particulate matter and possible bacterial or other contaminant that would interfere with the assay.

B. Mutagenicity Assay

The dose levels selected for the mutagenicity assay were 3.00, 2.00, 1.00, 0.500, 0.200, and 0.100 ml of unconcentrated test article per plate in the presence and absence of S9, as specified by the New Jersey Department of Environmental Protection. The mutagenicity assay results for Carbon Treated Water are presented in Tables 1 and 2. These data were generated in Experiment 14546-B1. The data are presented as mean revertants per plate ± standard deviation for each treatment and control group (Table 2) and as individual plate counts (Table 1).

In Experiment 14546-B1, all data were acceptable and no positive increases in the number of histidine revertants per plate were observed.

All criteria for a valid study were met.

CONCLUSIONS

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for Direct Water Samples indicate that under the conditions of this study, Johns Hopkins University's test article, Carbon Treated Water, did not cause a positive increase in the number of histidine revertants per plate with tester strains TA98 and TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

SECTION V. DATA TABLES

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TABLE 1

INDIVIDUAL PLATE COUNTS

TEST ARTICLE ID: Carbon Treated Water

EXPERIMENT ID: 14546-B1 VEHICLE: Sterile deionized water

DATE PLATED: 02-May-91 DATE COUNTED: 09-May-91

DOSE/PLATE		REVERTANTS PER PLATE TA98 1	TA98 2	TA98 3	TA100 1	TA100 2	TA100 3	BACKGROUND LAWN*
MICROSOMES: Rat Liver								
NEGATIVE CONTROL		36	27		117	107		1
VEHICLE CONTROL		27	27	23	157	132	103	1
TEST ARTICLE	0.100 ml	36	23		NC	NC		1
	0.200 ml	34	33		NC	NC		1
	0.500 ml	35	27		110	93		1
	1.00 ml	29	26		139	123		1
	2.00 ml	37	30		122	115		1
	3.00 ml	29	26		119	110		1
POSITIVE CONTROL **		1131	959		765	751		1
MICROSOMES: None								
NEGATIVE CONTROL		25	16		86	75		1
VEHICLE CONTROL		22	12	12	117	92	86	1
TEST ARTICLE	0.100 ml	15	12		97	89		1
	0.200 ml	23	23		115	104		1
	0.500 ml	19	17		117	105		1
	1.00 ml	17	14		129	109		1
	2.00 ml	16	13		93	89		1
	3.00 ml	15	13		113	108		*
POSITIVE CONTROL ***		119	165		471	463		1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal 2 = slightly reduced 3 = moderately reduced
4 = extremely reduced 5 = absent 6 = obscured by precipitate
sp = slight precipitate mp = moderate precipitate (requires hand count) hp = heavy precipitate (requires hand count)

NC = No count due to procedural error, plates did not receive an aliquot of tester strain.

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TABLE 2

SUMMARY OF TEST RESULTS

TEST ARTICLE ID: Carbon Treated Water

EXPERIMENT ID: 14546-B1

VEHICLE: Sterile deionized water

DATE PLATED: 02-May-91

DATE COUNTED: 09-May-91

	DOSE/PLATE	MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION TA98 MEAN	TA98 S.D.	TA100 MEAN	TA100 S.D.	BACKGROUND LAWN*
MICROSOMES: Rat Liver						
NEGATIVE CONTROL		32	6	112	7	1
VEHICLE CONTROL		26	2	131	27	1
TEST ARTICLE	0.100 ml	30	9	NC	-	1
	0.200 ml	34	1	NC	-	1
	0.500 ml	31	6	102	12	1
	1.00 ml	28	2	131	11	1
	2.00 ml	34	5	119	5	1
	3.00 ml	28	2	115	6	1
POSITIVE CONTROL **		1045	122	758	10	1
MICROSOMES: None						
NEGATIVE CONTROL		21	6	81	8	1
VEHICLE CONTROL		15	6	98	16	1
TEST ARTICLE	0.100 ml	14	2	93	6	1
	0.200 ml	23	0	110	8	1
	0.500 ml	18	1	111	8	1
	1.00 ml	16	2	119	14	1
	2.00 ml	15	2	91	3	1
	3.00 ml	14	1	111	4	1
POSITIVE CONTROL ***		142	33	467	6	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

NC = No count due to procedural error, plates did not receive an aliquot of tester strain.

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a CORNING Laboratory Services Company

MUTAGENICITY TEST ON

AN EXTRACT OF THE WATER SAMPLE

CARBON TREATED WATER

IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY (AMES TEST) MODIFIED FOR XAD-2 RESIN EXTRACTS

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Hazleton Washington, Inc.
5516 Nicholson Lane
Kensington, Maryland 20895

LABORATORY PROJECT ID

HWA Study No.: 14546-1-401X

SUBMITTED TO

Johns Hopkins University
Applied Physics Laboratory
4800 Atwell Road
Shady Side, MD 20764

STUDY COMPLETION DATE

June 12, 1991

United States • Germany • Japan • France • United Kingdom
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HAZLETON
WASHINGTON

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts

STUDY NO.: 14546-1-401X

PROTOCOL NO.: 401X EDITION NO.: 17

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection - Date	Findings Reported	Auditor
Preparation of S9 Mix - 05/15/91	05/15/91	D. Wallace
Final Report Review - 06/12/91	06/12/91	P. Postal

Patti Postal 6·12·91

Quality Assurance Unit Date Released

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COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160). To the best of the signer's knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The stability of the test article under the conditions of administration was the responsibility of the Sponsor. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of the HW[illegible]sion of Molecular and Cellular Services, 5516 Nicholson Lane, Kensingt[illegible]aryland 20895. After two years, these records will be transferred to permanent archives at Hazleton Washington, Vienna, Virginia.

SUBMITTED BY:

Study Director:

Timothy E. Lawlor, M.A.
Microbial Mutagenesis
Department of Molecular and Cellular Toxicology

6.12.91
Study Completion Date

HAZLETON
WASHINGTON

TABLE OF CONTENTS

SECTION I. SUMMARY

INTRODUCTION AND CONCLUSIONS

SUMMARY

A. Introduction

At the request of Johns Hopkins University, Hazleton Washington, Inc. examined an extract of the water sample, Carbon Treated Water, for mutagenic activity in the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver.

The tester strains used in this study were TA98 and TA100. The assay was conducted using two plates per dose level in the presence and absence of microsomal enzymes. Six dose levels of the test article were tested, from 100 to 2.49 μg per plate in both the presence and absence of S9. Vehicle controls (three plates per dose), negative controls, resin controls and positive controls were plated as part of the assay.

The doses tested in the mutagenicity assay were selected based on the amount of extractable organics recovered from the test article.

B. Conclusions

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts indicate that under the conditions of this study, an extract of Johns Hopkins University's test article, Carbon Treated Water, did not cause a positive increase in the number of histidine revertants per plate with tester strain TA98 or TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver.

SECTION II. STUDY INFORMATION

STUDY INFORMATION

A. Sponsor: Johns Hopkins University

B. Test Article: Carbon Treated Water

1. Physical Description, Test Article: clear colorless liquid

2. Date Received: 05/01/91

3. Physical Description, Extract: pale amber liquid

C. Type of Assay: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts

1. Protocol Number: HWA Protocol 401X, Edition 17

2. HWA Study Number: 14546-1-401X

D. Study Dates

1. Study Initiation Date: 05/01/91

2. Experimental Start: 05/15/91

3. Experimental Termination: 05/20/91

E. Study Personnel

Study Director: Timothy E. Lawlor, M.A.

Laboratory Supervisor: Michael S. Mecchi, B.S.

Research Assistant: Ralph S. McCrea, B.S.

Technician: Sow Hoong Hon, B.S.

SECTION III. MATERIALS AND METHODS

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames et al (1975).

MATERIALS

A. Tester Strains

The tester strains used were the Salmonella typhimurium histidine auxotrophs TA98 and TA100 as described by Ames et al (1975). The specific genotypes of these strains are shown in Table 1.

TABLE 1. TESTER STRAIN GENOTYPES

Histidine Mutation		Additional Mutations		
hisG46	hisD3052	LPS	Repair	R Factor
TA100	TA98	rfa	uvrB	+R

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the uvrB gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strain TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototropy) by frameshift mutagens. Tester strain TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

1. Source of Tester Strains

The tester strains in use at HWA were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

2. Storage of the Tester Strains

a. Frozen Permanent Stocks

Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (approximately 0.5 - 1.5 ml) at ≤-70°C.

b. Master Plates

Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 μM), biotin (3 μM), and for strains containing the R-factor, ampicillin (25 μg/ml). Tester strain master plates were stored at 5 ± 3°C.

3. Preparation of Overnight Cultures

a. Inoculation

Overnight cultures for use in all testing procedures were inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 ± 25 rpm; incubation, 37 ± 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began.

b. Harvest

To ensure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring of culture turbidity. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance (%T) reading on a spectrophotometer. This target turbidity ensures that cultures have reached a density of at least 0.5 X 10^9 cells per ml and that the cultures have not overgrown. Overgrown (stationary) cultures may exhibit decreased sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached and were placed at 5 ± 3°C.

4. Confirmation of Tester Strain Genotypes

Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

a. rfa Wall Mutation

The presence of the rfa wall mutation was confirmed by demonstration of the cultures sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

b. pKM101 Plasmid R-factor

The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

c. Characteristic Number of Spontaneous Revertants

The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with the appropriate vehicle on selective media.

5. Tester Strain Media

a. Culturing Broth

The broth used to grow overnight cultures of the tester strains was Vogel-Bonner salt solution supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

b. Agar Plates

Bottom agar (25 ml per 15 x 100 mm petri dish) was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (W/V) agar and 0.2% (W/V) glucose.

c. Overlay Agar for Selection of Histidine Revertants

Overlay (top) agar was prepared with 0.7% agar (W/V) and 0.5% NaCl (W/V) and was supplemented with 10 ml of 0.5 mM histidine/biotin solution per 100 ml agar. When S9 mix is required, 2.0 ml of the supplemented top agar is used in the overlay. However, when S9 is not required, water is added to the supplemented top agar (0.5 ml of water per 2 ml of supplemented top agar) and the resulting 2.5 ml of diluted supplemented top agar is used for the overlay. This dilution ensures that the final top agar and amino acid

supplement concentrations remain the same both in the presence and absence of S9.

B. Liver Microsomal Enzyme Reaction Mixture (S9 Mix)

1. S9 Homogenate

Liver microsomal enzymes (S9 homogenate) were purchased from Molecular Toxicology, Inc., Annapolis, MD 20401, Batch 0327 (37.8 mg of protein per ml). The homogenate was prepared from male Sprague-Dawley rats that had been injected (i.p.) with Aroclor™ 1254 (200 mg per ml in corn oil) at 500 mg/kg as described by Ames et al, 1975.

2. S9 Mix

The S9 mix was prepared immediately prior to its use in any experimental procedure. The S9 mix contained the components indicated in Table 2.

TABLE 2. S9 MIX COMPONENTS

H_2O	0.70 ml
1M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 ml
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 ml
0.825M KCl/0.2M $MgCl_2$	0.04 ml
S9 Homogenate	0.10 ml
	1.00 ml

C. Controls

1. Vehicle Controls

Dimethylsulfoxide (DMSO, CAS# 67-68-5, Sigma Chemical Co., Lot 30H0608, 99+%) vehicle controls were plated for both tester strains in the presence and absence of S9. The vehicle control was plated, using a 50 µl aliquot of DMSO (equal to the maximum aliquot of test article dilution plated), along with a 100 µl aliquot of the appropriate tester strain and a 500 µl aliquot of S9 (when necessary), on selective agar.

2. Negative Controls

Negative controls were plated for both tester strains in the presence and absence of S9. The negative controls consisted of a 100 µl aliquot of the appropriate tester strain and S9 (when appropriate) plated on selective agar.

3. Resin Controls

Resin controls were plated for both tester strains in the presence and absence of S9. A sample of deionized water was extracted and concentrated using the same XAD-2 resin adsorption procedure used for the test article. The resin control was tested at a single concentration. The resin controls consisted of a 50 µl aliquot of the resin control extract, a 100 µl aliquot of tester strain and an aliquot of S9 (when appropriate) plated on selective agar.

4. Positive Controls

The combinations of positive controls, activation condition and tester strains plated concurrently with the assay are indicated in Table 3.

TABLE 3. POSITIVE CONTROLS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 µg
TA98	-	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	-	sodium azide	2.0 µg

a. Source and Grade of Positive Control Articles

2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade; 2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98%; sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade.

5. Sterility Controls

a. S9 Mix

The S9 mix was checked for sterility by plating 0.5 ml on selective agar.

METHODS

A. Mutagenicity Assay

1. Design

The assay was performed using tester strains TA98 and TA100 both in the presence and absence of S9 mix. Six dose levels of the test

article extract were tested along with the appropriate vehicle, negative, resin and positive controls. The dose levels tested were selected based on the amount of extractable organics recovered in the extraction procedure.

2. Frequency and Route of Administration

The tester strains were exposed to the test article via the plate incorporation methodology originally described by Ames et al (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. In the plate incorporation methodology, the test article, the tester strain and the S9 mix (where appropriate) are combined in molten agar which is overlaid onto a minimal agar plate. Following incubation at 37 ± 2°C for 48 ± 8 hr, revertant colonies will be counted. All dose levels of test article, negative controls, resin controls and positive controls were plated in duplicate, and the vehicle controls were plated in triplicate.

B. Plating Procedures

Each plate was labeled with a code which identified the test article, test phase, tester strain, activation condition and dose level. The S9 mix and dilutions of the test article were prepared immediately prior to their use.

When S9 mix was not required, 100 µl of tester strain and 50 µl of vehicle or test article dose was added to 2.5 ml of molten selective top agar (maintained at 45 ± 2°C). When S9 mix was required, 500 µl of S9 mix, 100 µl of tester strain and 50 µl of vehicle or test article dose was added to 2.0 ml of molten selective top agar. After the required components had been added, the mixture was vortexed and overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 hr at 37 ± 2°C. Positive controls and the resin control were plated using a 50 µl plating aliquot.

C. Scoring the Plates

Plates which were not evaluated immediately following the incubation period were held at 5 ± 3°C until such time that colony counting and bacterial background lawn evaluation could take place.

1. Bacterial Background Lawn Evaluation

The condition of the bacterial background lawn was evaluated for evidence of cytotoxicity and test article precipitate. Evidence of cytotoxicity was scored relative to the vehicle control plate and was recorded along with the revertant counts for all plates at that dose level on the data tables using the code system presented at the end of the Materials and Methods Section.

2. Counting Revertant Colonies

The number of revertant colonies per plate for the vehicle controls and all plates containing test article were counted manually. The number of revertant colonies per plate for the positive controls were counted by automated colony counter.

D. Analysis of Data

For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The results of these calculations are presented in tabular form in the Data Tables Section of this report.

EVALUATION OF TEST RESULTS

Before assay data can be evaluated, the criteria for a valid assay must be met.

A. Criteria For A Valid Assay

The following criteria are used to determine a valid assay:

1. Tester Strain Integrity

a. rfa Wall Mutation

To demonstrate the presence of the rfa wall mutation, tester strain cultures must exhibit sensitivity to crystal violet.

b. pKM101 Plasmid

To demonstrate the presence of the R-factor plasmid, pKM101, cultures of tester strains TA98 and TA100 must exhibit resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants

To demonstrate the requirement for histidine, the tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable ranges for the vehicle controls are as follows:

TA98	8 - 60
TA100	60 - 240

d. Tester Strain Culture Density

To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures must be greater than or equal to 0.5×10^9 bacteria per ml and/or have reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 0.5×10^9 bacteria per ml.

e. Positive Control Values

(1) Positive Control Values in the Absence of S9 Mix

To demonstrate that the tester strains are capable of identifying a mutagen, the mean value of a positive control for a respective tester strain must exhibit at least a 3-fold increase over the mean value of the vehicle control for that strain.

(2) Positive Control Values in the Presence of S9 Mix (S9 Mix Integrity)

To demonstrate that the S9 mix is capable of metabolizing a promutagen to its mutagenic form(s), the mean value of the positive control for a respective tester strain in the presence of the S9 mix must exhibit at least a 3-fold increase over the mean value of the vehicle control for that strain.

An acceptable positive control in the presence of S9 for a specific strain will be evaluated as having demonstrated both the integrity of the S9 mix and the ability of the tester strain to detect a mutagen.

2. Cytotoxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data.

B. Criteria For A Positive Response

Once the criteria for a valid assay have been met, responses observed in the assay are evaluated as follows:

1. Tester Strains TA98 and TA100

For a test article to be considered positive, it must produce at least a 2-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

RECORDS TO BE MAINTAINED

All raw data, reports, protocols and modifications will be maintained by the Department of Molecular and Cellular Toxicology of HWA for a period of up to two years following submission of the final report to the Sponsor. After two years, all raw data and reports will be transferred to the HWA archives for permanent storage.

REFERENCES

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Maron, D.M., and Ames, B. Revised Methods for the Salmonella Mutagenicity Test. Mutation Research 113:173-215 (1983).

Vogel, H.J., and D.M. Bonner. Acetylornithinase of E. coli: Partial Purification and Some Properties, J. Biol. Chem. 218:97-106 (1956).

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS OF BACKGROUND LAWN
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

SECTION IV. RESULTS AND CONCLUSIONS

RESULTS

A. Test Article Handling

The water sample, Carbon Treated Water, was held at 5 ± 3°C until extracted and concentrated by the XAD-2 resin adsorption as described in the "Environmental Water Sample Processing Protocol No. 12, October, 1989."

The test article (36 liters) was passed through XAD-2 resin and solvent exchanged to dimethylsulfoxide (DMSO) using the procedure described below. Deionized water (48 liters) was also processed in the same manner as an internal control for the extraction procedure (resin control).

A chromatographic column (22 mm I.D.) was slurry-packed with 15 g of XAD-2 resin and washed with acetone (CAS# 67-64-1, Fisher Scientific, Lot 900324) and water. All extraction procedures were performed at 5 ± 3°C. The test article was passed through the XAD-2 column using gravity flow at a rate of 60 to 80 drops/min. Material adsorbed to the column was eluted with acetone and methylene chloride (CAS# 75-09-2, Fisher Scientific, Lot 890625). The eluate was reduced in volume using a rotary evaporator. Duplicate aliquots of the concentrate were transferred to tared aluminum weighing dishes, the solvent allowed to evaporate off, and the amount of material extracted from the water samples determined gravimetrically. The remainder of the extract was solvent exchanged into 0.5 ml dimethylsulfoxide (DMSO, CAS# 67-68-5, Sigma Chemical Co., Lot 30H0608, 99+%).

Extractable Organics

	Sample Volume	Extractives mg/0.5 ml
Carbon Treated Water	36 Liters	2.7
Resin Control (deionized water)	48 Liters	0.0

B. Dose Selection

Since the amount of the sample available for the assay was limited, no dose rangefinding study was performed on this sample. Routinely, between 50 to 75% of the available extract will be used in the initial mutagenicity assay, with the remaining extract reserved for possible retesting and confirmation studies. The total amount of extractives obtained after processing 36 liters of the sample through XAD-2 resin was 2.7 mg in a volume of 0.5 ml DMSO. The deionized water (48 liters) passed through XAD-2 resin had a total extractives of 0.0 mg in a volume of 0.5 ml DMSO. The amount of the extractives obtained from the deionized water control was deducted from the total extractives of the test sample (2.7 mg - 0.0 mg = 2.7 mg) to

calculate the doses used in the mutagenicity assay. Based on the amount of available extractives, the test sample extract was assayed at six doses, 100, 50.0, 24.9, 10.0, 5.00 and 2.49 μg of extractives per plate using two plates per dose level.

C. Mutagenicity Assay

The dose levels selected for the mutagenicity assay ranged from 100 to 2.49 μg per plate in both the presence and absence of S9.

The mutagenicity assay results for Carbon Treated Water are presented in Tables 1 and 2. These data were generated in Experiment 14546-B1. The data are presented as mean revertants per plate ± standard deviation for each treatment and control group (Table 2) and as individual plate counts (Table 1).

In Experiment 14546-B1 (Tables 1 and 2), all data were acceptable and no positive increases in the number of histidine revertants per plate were observed with tester strains TA98 or TA100 in either the presence or absence of S9.

All criteria for a valid study were met.

CONCLUSIONS

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) Modified for XAD-2 Resin Extracts indicate that under the conditions of this study, an extract of Johns Hopkins University's test article, Carbon Treated Water, did not cause a positive increase in the number of histidine revertants per plate with tester strains TA98 and TA100 either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver.

SECTION V. DATA TABLES

HAZLETON
WASHINGTON

TABLE 1

INDIVIDUAL PLATE COUNTS

TEST ARTICLE ID: Carbon Treated Water

EXPERIMENT ID: 14546-B1

DATE PLATED: 15-May-91 VEHICLE: DMSO

DATE COUNTED: 20-May-91 PLATING ALIQUOT: 50 µl

DOSE/PLATE	REVERTANTS PER PLATE TA98 1	TA98 2	TA98 3	TA100 1	TA100 2	TA100 3	BACKGROUND LAWN*
MICROSOMES: Rat Liver							
RESIN CONTROL	27	37		176	135		1
NEGATIVE CONTROL	39	54		104	115		1
VEHICLE CONTROL	39	25	51	88	82	110	1
TEST ARTICLE 2.49 µg	46	28		116	135		1
5.00 µg	41	44		113	129		1
10.0 µg	41	36		127	133		1
24.9 µg	42	48		148	109		1
50.0 µg	52	39		123	131		1
100 µg	37	47		114	162		1
POSITIVE CONTROL **	1016	1036		1195	1239		1
MICROSOMES: None							
RESIN CONTROL	15	10		98	95		1
NEGATIVE CONTROL	21	10		105	88		1
VEHICLE CONTROL	13	19	23	86	75	103	1
TEST ARTICLE 2.49 µg	14	16		79	95		1
5.00 µg	28	28		88	93		1
10.0 µg	16	26		98	100		1
24.9 µg	24	29		114	97		1
50.0 µg	20	33		103	88		1
100 µg	28	26		87	111		1
POSITIVE CONTROL ***	112	115		350	333		1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal 2 = slightly reduced 3 = moderately reduced
4 = extremely reduced 5 = absent 6 = obscured by precipitate
sp = slight precipitate mp = moderate precipitate (requires hand count) hp = heavy precipitate (requires hand count)

HAZLETON
WASHINGTON

TABLE 2

SUMMARY OF TEST RESULTS

TEST ARTICLE ID: Carbon Treated Water

EXPERIMENT ID: 14546-B1

DATE PLATED: 15-May-91 VEHICLE: DMSO

DATE COUNTED: 20-May-91 PLATING ALIQUOT: 50 µl

	DOSE/PLATE	MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION TA98 MEAN	TA98 S.D.	TA100 MEAN	TA100 S.D.	BACKGROUND LAWN*
MICROSOMES: Rat Liver						
RESIN CONTROL		32	7	156	29	1
NEGATIVE CONTROL		47	11	110	8	1
VEHICLE CONTROL		38	13	93	15	1
TEST ARTICLE	2.49 µg	37	13	126	13	1
	5.00 µg	43	2	121	11	1
	10.0 µg	39	4	130	4	1
	24.9 µg	45	4	129	28	1
	50.0 µg	46	9	127	6	1
	100 µg	42	7	138	34	1
POSITIVE CONTROL **		1026	14	1217	31	1
MICROSOMES: None						
RESIN CONTROL		13	4	97	2	1
NEGATIVE CONTROL		16	8	97	12	
VEHICLE CONTROL		18	5	88	14	1
TEST ARTICLE	2.49 µg	15	1	87	11	1
	5.00 µg	28	0	91	4	1
	10.0 µg	21	7	99	1	1
	24.9 µg	27	4	106	12	1
	50.0 µg	27	9	96	11	1
	100 µg	27	1	99	17	1
POSITIVE CONTROL ***		114	2	342	12	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal 2 = slightly reduced 3 = moderately reduced
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